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THE BERNARD BECKER

Friedenwald Memorial

PROCEEDINGS

of the

Association for Research in Ophthalmology, Inc.

Twenty-seventh Meeting

San Francisco, California

June 23-26, 1958

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BERNARD BECKER, M.D.

REMARKS ON ACCEPTANCE OF FRIEDENWALD MEMORIAL PLAQUE

Bernard Becker, M.D.
Saint Louis, Missouri

It is indeed a privilege and an honor to present the Friedenwald Memorial Lecture. However, I do so only as a representative of a research team. On behalf of my co-workers and myself, may I thank the committee for this recognition. I am also grateful for the opportunity to express my deep feelings for Jonas Friedenwald and my appreciation for the years I was fortunate enough to enjoy his friendship and guidance. His personal charm, brilliance, and accomplishments altered the lives and thoughts of men with varied interests in a wide variety of disciplines. Few were more influenced than I .

My first visit to Baltimore in 1946 was for the purpose of seeking aid from Dr. Friedenwald on a difficult histochemical problem. I came unaware that he was anything more than an outstanding expert in enzymatic histochemistry. After an entire afternoon of conversation and sightseeing, I left entranced by a few of Dr. Friedenwald's favorite subjects—Baltimore, Hopkins, and the potentialities afforded by the eye as a research tool. Shortly thereafter, all previous plans were altered and I came to the Wilmer Institute to work in Friedenwald's laboratory. There, largely through the efforts of Friedenwald and Alan Woods, I came to appreciate the academic and research aspects and exciting opportunities of ophthalmology.

Throughout my research and clinical training in ophthalmology, and even after my move to Saint Louis, Jonas Friedenwald continued to provide sympathetic understanding, critical appraisal, suggestions for new and ingenious approaches, and an enormous fund of knowledge. Hour upon hour of discussions of data and plans for experiments were often followed by long evening telephone conversations or lengthy penciled correspondence. Week-ends, vacations, distance, and even illness failed to interfere with this steady stream of exciting ideas, new interpretations, and inspiring concepts during the all-too-brief nine years that I knew Jonas Friedenwald. We shared the occasional excitement of successful experiments and the frequent frustrations of failures. Much of the research task is accomplished when one has Friedenwald's ability to formulate the right questions. His scientific acumen and intuitive ingenuity are sorely missed by many of us today.

The widespread interests and broad full life of Jonas Friedenwald have been amply described by such experts as Alan C. Woods, Felix Frankfurter, and Abel Wolman. Many highly specialized authorities considered their specialty as Friedenwald's primary concern. His multiple interests and activities are recalled by innumerable less known instances and episodes. Long letters written by hand while on vacation contained pages of formulas interspersed with beautiful descriptive passage of his natural surroundings or amusing sketches of his animal friends. Learned discussions in the living room might be interrupted by the entry of one of the children asking for Jonas' demonstration of a hippopotamus in the act of reclining on the floor. In spite of overwhelming demands upon his time as an astute clinician, capable administrator, sympathetic advisor, and inspiring teacher, he was never too busy for the individual, particularly the young student. In addition, he managed to find time to operate upon animals, to serve as a guinea pig for tonography and steroid experiments, and to delight in building equipment in his beloved workshop. Work on tonometer calibrations, kinetics of enzymatic activity, endocrinologic aspects of the adrenal cortex in diabetes, ocular and gastric secretory mechanisms, and enzymatic histochemistry were carried out simultaneously with the writing of newspaper

editorials and the development of new theories of the nature of binocular visual space and its application to perspective in painting through the ages. Throughout, his love of people, his modest unassuming manner, and his dry subtle humor won him the respect and affection of his peers, his co-workers, and his students.

That attribute of Jonas Friedenwald that I would select as most outstanding and worthy of emulation was his life as the perpetually receptive student. He had an uncanny capacity for rapid retention. On many occasions the information sought, the solution of a laboratory problem, or the development of far-reaching concepts, resulted from his ability to recall data, patients, ideas, or the appropriate literature or expert that he had seen or heard days, months, or even years before. He advocated the "textbook under the tree" and "learn by doing" approach to the acquisition of basic knowledge. Stimulated by ideas, experimental results, patients, or literature, it was his conviction that any unfamiliar methodology or discipline could be learned readily and applied to the problem at hand. This liberal approach to education and the atmosphere of academic freedom which pervaded his laboratory made it a fertile ground for scientific productivity. This was an important part of the genius that was Friedenwald.

Much of what is presented at a research association meeting today rests on the solid foundations established by Friedenwald. In addition his profound comments provide a fundamental background for a number of the practical and philosophic problems confronting the research worker, the educator, the administrator, and the clinician today. In this fashion his spirit continues with us.

640 South Kingshighway (10).

BIOGRAPHY: BERNARD BECKER, M.D.

In 1947, Dr. Jonas Friedenwald formally announced to his colleagues that a brilliant young man would soon make his presence felt in the ophthalmologic world. Dr. Bernard Becker has so quickly fulfilled this prophecy that even this early in his career the problem is not how to find enough material to fill a biography, but what to exclude to prevent its being too long. At 37, this represents a considerable achievement.

He was born in Brooklyn on August 21, 1920. In 1937, following graduation from a public high school in Brooklyn, he attended Princeton University. Majoring in chemistry, he did a significant piece of work during his junior year on the variations of fluid viscosity with changes in temperature. In his senior year he became interested in the molecular structure of proteins; this led to studies of nylon and wool fibers, involving the construction of elaborate models. His work won him the annual prize in physical chemistry. In 1941, after leading his class for four years, he graduated summa cum laude, carrying away the scholarship given for postgraduate work at Harvard.

During the next summer, he worked at the Bureau of Standards in Washington, D.C., where he studied stress-strain curves of protein and plastic fibers, and the underlying intra- and intermolecular mechanisms involved.

He went on to Harvard Medical School, graduating in 1944 with honors. The Mount Sinai Hospital in New York City claimed him for nine months of rotating internship, following which he entered the U. S. Army. He served a 28-month tour of duty in various induction and separation centers, as an army psychiatrist. Part of his tour was spent in Washington, D.C., where, having become interested in histochemistry, he attended a lecture given by Dr. Friedenwald. After the lecture he talked to Dr. Friedenwald and was

subsequently invited to Baltimore, where their mutual interests led Dr. Friedenwald to offer him a position in his laboratory at the Wilmer Institute. Although ophthalmology had not been his major interest, because of his fascination for histochemistry and his liking for Dr. Friedenwald, he became sufficiently interested to apply for the residency training program. In 1948, after working for one year in the laboratory, he became a member of the Wilmer Institute house staff.

In 1947, he learned of the periodic-acid fuchsin stain for the histologic demonstration of mucoids. Being interested in the mucoids of the vitreous, he attempted to use this stain on sections of the eye. Seeing these slides, Dr. Friedenwald suggested that a nice delineation of the vascular pattern of the retina might be obtained by making flat preparations of the retina. He set Dr. Becker to work, devising a method of making flat preparations and modifying the Hotchkiss-McManus stain for better visualization of the vessels.

Dr. Friedenwald had hypothesized that the apparent aneurysmal dilatations of blood vessels seen in diabetics by benzidine staining of red cells were in reality endothelialized, small hemorrhages. With these superior techniques, he was easily convinced that the lesions were indeed aneurysms.

During the next two years, Dr. Becker pursued further work on diabetes and other retinal vascular pathology, including work on the association of aneurysms with clinical and experimental retinal vein occlusions. It was at this time also that Dr. Becker presented a paper implicating the adrenal glands in the etiology of diabetic retinopathy.

In 1950, he worked for a number of weeks with Ernst Bárány and Eric Linnér in Uppsala, Sweden, on the rate of aqueous secretion, the alterations of ciliary blood flow, and the effects of ACTH on aqueous humor ascorbate. From there, he went to the Carlsberg Laboratories in Copenhagen, where he spent several weeks studying microchemical techniques. He ended by visiting most of the major eye clinics in Europe, from which he carried away much useful experience.

He returned to Hopkins to complete his residency and to continue his work on aqueous flow, tonography, and mechanisms of aqueous secretion (see Bibliography). During this period he also made probably his best known, single contribution: the discovery of the effects of Diamox on intraocular pressure. After his training, he remained on the staff of the Wilmer Institute, as an assistant professor. Six months later, be became the Head of the Department of Ophthalmology at the Washington University School of Medicine in Saint Louis. Here, despite extremely time-consuming administrative and teaching duties, and an active consultation practice, he has continued his work as chief of the research laboratores. When he came to Saint Louis, the research budget was small, and there were two full-time staff men. The research budget has grown in a period of four years, and there are now eight members of the full-time staff. The house-staff now numbers 16 as contrasted with six formerly. Under his expert guidance, a great deal of significant work continues to come out of the department.

Outside the Department of Ophthalmology, Dr. Becker is currently active on many committees: he has served for four years on the Sensory Diseases Study Section of the National Institutes of Health, and he is also a member of the Ophthalmology Training Grants Committee and a consultant to the Glaucoma Detection Program of the same organization; he serves on the National Committee for Research in Ophthalmology, the Scientific Advisory Committee of the National Council to Combat Blindness, and the Glaucoma Committee of the National Society for the Prevention of Blindness. He is on the Tonometer Calibration Committee of the American Academy of Ophthalmology and Otolaryngology, on the editorial staff of THE AMERICAN JOURNAL OF OPHTHALMOLOGY, and has for three years written the glaucoma annual reviews for the *A.M.A. Archives of*

Ophthalmology. He has participated in the Macy Foundation Conference on Glaucoma, and he is currently president of the Washington University Medical Society. He has presented the Schneider Foundation Lecture, the Mark Schoenberg Lecture, and he has received the Edward Lorenzo Holmes Award.

In 1950, he married Janet Rosenwald, from Philadelphia. He and Janet have four children: Diana Joan, age six; Stephen Jonas, age five; John Lawrence, age four; and Bernard, Jr., age two. Steve, of course, is named for Jonas Friedenwald, who, at the time of his birth, sent him an autographed copy of the Armed Forces Institute *Atlas of Pathology*, "to be kept in escrow" for him because "he came off the press simultaneously with this book."

The demands on Dr. Becker's time are beyond belief. He is active on medical school and hospital committees and serves in an advisory capacity for a number of local organizations. He has so many invitations to speak that he has been forced to make a rule to speak nowhere, except under the rarest circumstances. However, he makes it a point to find relaxation by swimming regularly, and takes great pride in the fishing at his country home. He has a great fund of knowledge. Every month he makes a series of trips to the library, to read all of the major journals published in other fields of medicine. He seems to know of new developments almost as soon as they occur. His knowledge of advances in other fields, his awareness of the latest thinking in his own, combined with his own remarkable inventiveness, give him an endless supply of research ideas. To have such a man around, is to keep a research department continually active and producing.

He is also a born teacher. He can talk at any level, and his discussion of a subject on the spur of the moment would suggest a week's careful preparation. His patience is endless. He has a way of walking into a laboratory and within a few minutes putting his finger on the crux of a problem, suggesting improvements in techniques and changes in approach. All this is done with a warmth and understanding which make the people who work in his laboratories completely devoted to him.

Unselfishness is characteristic of him, and he is jealous only of his research time. He will give unstintingly of his time to anything which furthers basic scientific knowledge. At least 10 years ago, he expressed himself unenamored of the honors that go to the successful scientist, and this is as true today as it was then. Such an attitude lends itself to objectivity; about him the spirit of competition fades into one of co-operation. This is the epitome of his philosophy of life.

Lawrence T. Post, Jr.

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CARBONIC ANHYDRASE AND THE FORMATION OF AQUEOUS HUMOR*

The Friedenwald Memorial Lecture

Bernard Becker, M.D.

Saint Louis, Missouri

In the Proctor Lecture on "The formation of the intraocular fluid" 10 years ago, Friedenwald[1] raised "the question as to whether more effort might not profitably be directed toward a reduction in the formation of aqueous in cases of glaucoma, in addition to the present approach which concerns largely an effort to increase the outflow of fluid from the eye." Research based largely upon the theories and groundwork described by Friedenwald in this lecture made his intuitive prediction a reality less than five years later.[2,3]

The usefulness or carbonic anhydrase inhibitors in ophthalmology has not been limited to the therapy of the glaucomas, for the availability of such agents as research tools has contributed greatly to our knowledge of aqueous humor dynamics. Not only did Friedenwald suggest a need for carbonic anhydrase in the secretion of aqueous humor, but he also provided us with a more complete theory of aqueous humor dynamics.[4] It is largely on the basis of these formulations as well as his work on standardization and cali-

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* From the Department of Ophthalmology and the Oscar Johnson Institute, Washington University School of Medicine.

bration of tonometers[5,6] that it has been possible to quantitate the secretory alterations induced by carbonic anhydrase inhibitors.

I. Carbonic anhydrase and its inhibition

The enzyme carbonic anhydrase (C.A.) catalyzes the reversible hydration of carbon dioxide:

$$CO_2 + H_2O \xrightleftharpoons{C.A.} H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

In addition to its presence in the red blood cell, it has been found in a number of secretory sites (ciliary body, gastric mucosa, pancreas, renal tubule, and so forth). The recent availability of highly active carbonic anhydrase inhibitors has stimulated great interest in the part played by this enzyme in several secretory processes, and has added to our meager understanding of the intricate problems of ion transport.

The most effective carbonic anhydrase inhibitors available are the sulfonamides, RSO_2NH_2. As Roblin* has postulated (fig. 1), the structure of the enzyme substrate (H_2CO_3) and sulfonamide portion of the inhibitor closely resemble one another. One may speculate that the inhibitor occupies the active site on the enzyme surface making it unavailable to the substrate. It is also apparent that substitutions on the amino nitrogen will alter the resemblance to the substrate and block its ability to occupy the postulated active site. Thus, nitrogen substituted sulfonamides (RSO_2NHR') are inactive as carbonic anhydrase inhibitors.[7,8] These compounds serve as excellent controls for the analogs which are active carbonic anhydrase inhibitors. In much of the recent work on secretion of aqueous the tertiary-butyl or other substituted inactive derivatives have been used in the control animals.[9,10]

Of the inhibitors (fig. 2) acetazolamide†

* Roblin, R. O.: Personal communication, 1958.

† Acetazolamide, methazolamide, CL 13,850, and CL 17,262 were furnished by the Lederle Laboratories Division, American Cyanamid Company; chlorothiazide and dichlorphenamide were supplied by Merck, Sharp, and Dohme; ethoxzolamide was obtained from Upjohn and Company.

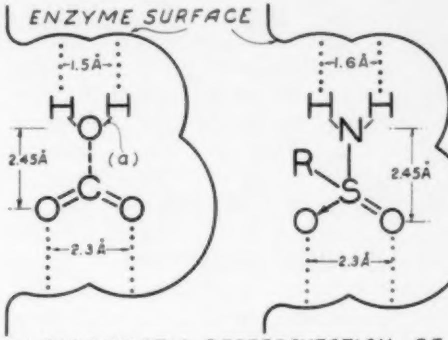
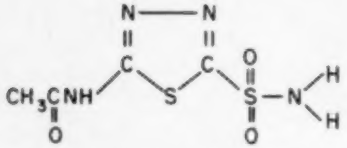


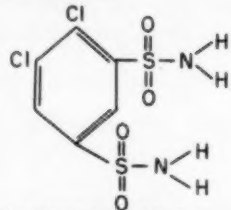
Fig. 1 (Becker). Schematic demonstration of the similar spatial relationship of carbonic acid and unsubstituted sulfonamides at the presumed active center of the enzyme carbonic anhydrase. I am grateful to Dr. R. O. Roblin of American Cyanamid Company, New York, for permission to use this unpublished diagram.

has received the most intensive study and largest clinical trial.[11] Such newer sulfonamides as dichlorphenamide and ethoxzolamide are more potent inhibitors of carbonic anhydrase. They appear to accomplish much the same decrease in intraocular pressure and aqueous secretion in animals and man as does acetazolamide but at lower dose levels. This is also true for their effects on the renal tubule of mammals as well as alligators.[12] It is understandable, but indeed unfortunate from the clinical point of view, that they have much the same systemic and side effects as acetazolamide even at the reduced dose level. On the other hand, methazolamide is only somewhat more active as an inhibitor of carbonic anhydrase than is acetazolamide.[14] However this agent penetrates the aqueous humor and spinal fluid some three to five times as readily as does acetazolamide. It is therefore effective in lowering intraocular pressure and in preventing experimental convulsions in mice at one third to one fifth the acetazolamide dosage.[15] At this lower dose level it demonstrates much less renal and systemic electrolyte alterations, but similar ocular and central nervous system

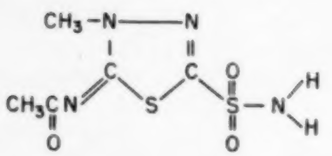
Sulfonamide Inhibitors of Carbonic Anhydrase



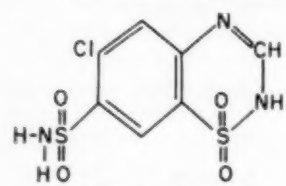
Acetazolamide (Diamox)



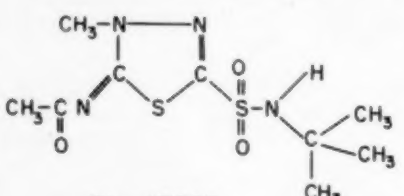
Dichlorphenamide (Daranide)



Methazolamide (Neptazone)

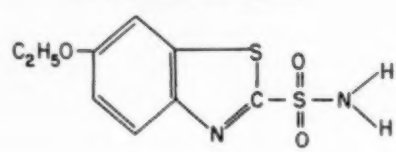


Chlorothiazide (Diuril)



CL – 17262

(Inactive Derivative of Neptazone)



Ethoxzolamide (Cardrase)

FIG. 2 (Becker). Structural formulas for carbonic anhydrase inhibitors (and the inactive analog of Neptazane) used in experiments included in this paper.

effects to those induced by other carbonic anhydrase inhibitors.

Chlorothiazide has much less activity as a carbonic anhydrase inhibitor and has been found not to alter intraocular pressure in man or animals. The tertiary-butyl derivatives of acetazolamide (CL 13,850) and methazolamide (CL 17,262) are completely inactive as inhibitors.[8]

II. Effect of carbonic anhydrase inhibitors on intraocular pressure and outflow facility

By various methods of measurement including manometry, applanation, and Schiøtz tonometry, it has been established beyond doubt that the systemic administration of potent carbonic anhydrase inhibitors lowers intraocular pressure. This ocular hypotensive action is most apparent in glaucomatous eyes. It is also demonstrated in the eyes of normal humans as well as such experimental animals as the rabbit, guinea pig, dog, cat, and monkey. In all species studied the fall in intraocular pressure is not associated with a decrease in the resistance to outflow of aqueous humor from the eye. These findings are therefore consistent with the postulated decrease in aqueous formation induced by systemic carbonic anhydrase inhibition.

Tonometry and tonography are subject to a number of assumptions and sources of error.[16, 17] In addition to technical difficulties and inaccuracies of instrumentation, these in-

clude problems of calibration, variations from the assumed average values for scleral rigidity and corneal curvature, changes in blood volume within the eye, alterations in episcleral venous pressure, and possible changes in secretory and outflow characteristics induced by the application of the tonometer. However, when suitable correction factors are applied for a number of these variables and the 1955 Friedenwald tables[6,18] are used, estimates of intraocular pressure and outflow facility are obtained from tonography which are in reasonable agreement with manometric and perfusion measurements.[19,20] The similarity of values obtained is much more impressive when comparison is made on the same eyes.[21] Many of the sources of error are largely avoided or compensated when one confines the studies to relative changes in aqueous formation rather than its absolute value. Moreover, the tonographic method has the enormous practical advantages of allowing repeated studies on the same eye and application to human eyes.

Using the equation:[22]

$$F = C(P_0 - P_v) \qquad (1)$$

where F is the rate of flow (μl/min.), P_0 the intraocular pressure (mm. Hg), P_v the episcleral venous pressure (mm. Hg), and C the outflow facility (μl/min./mm. Hg), one may obtain estimates of rate of formation of aqueous humor in the individual eye. Repeating the tonographic tracing after the administration of a given drug permits an evaluation of the effects of the agent used on aqueous secretion as well as on outflow facility.

Assuming no significant change in episcleral venous pressure[23] the average tonographic changes in rate of aqueous flow induced by systemic carbonic anhydrase inhibition are presented in Table 1. Included are data on the effects of such carbonic anhydrase inhibitors as acetazolamide, dichlorphenamide, methazolamide, and ethoxzolamide on rabbit and human eyes. It is apparent that these effective carbonic anhydrase inhibitors all result in some 45 to 55-percent inhibition of aqueous secretion in man and 50 to 60-percent suppression in rabbits. The inactive analogs of acetazolamide and methazolamide have been used in rabbits as controls. No significant alteration in rate of flow follows their administration. Chlorothiazide, although originally described as a potent carbonic anhydrase inhibitor,[24] was found to be ineffective in lowering intraocular pressure or decreasing aqueous formation in either species. Subsequently, it has been demonstrated to have much less carbonic anhydrase inhibiting activity than initially postulated.

TABLE 1

TONOGRAPHIC ESTIMATES OF THE AVERAGE ALTERATION IN FLOW (ΔF) INDUCED BY CARBONIC ANHYDRASE INHIBITORS

Carbonic Anhydrase Inhibitor	Man		Rabbit	
	No. Eyes	ΔF	No. Eyes	ΔF
Acetazolamide[b]	126	−52%	116	−58%
Dichlorphenamide[a]	20	−48%	40	−50%
Methazolamide[a]	64	−46%	26	−53%
Ethoxzolamide[c]	50	−45%	—	—
Chlorothiazide[d]	16	− 2%	32	− 6%
*CL 17, 262[a]	—	—	36	+ 4%
†CL 13, 850[b]	—	—	10	− 5%

[a] Dose for rabbits 20 mg./kg. intravenously—for man 100 mg. orally.
[b] Dose for rabbits 100 mg./kg. intravenously—for man 500 mg. orally.
[c] Dose for man 250 mg. orally.
[d] Dose for rabbits 20–30 mg./kg. intravenously—for man 500 mg. orally.
* Inactive tertiary-butyl derivative of methazolamide.
† Inactive tertiary-butyl derivative of acetazolamide.

From equation (1) it is apparent that the fall in intraocular pressure will be greater in glaucomatous eyes and very much less in normal eyes. The decrease in pressure is further limited in normal eyes of rabbits and man by compensatory decreases in outflow facility, thus tending to maintain intraocular pressure in spite of the decreased rate of aqueous formation.[25] This state of "pseudo-resistance" to the pressure-lowering effects of carbonic anhydrase inhibitors is now well recognized. Fortunately, glaucomatous eyes rarely possess this capacity.

III. Effect of carbonic anhydrase inhibition on the composition of aqueous humor

The study of the composition of the aqueous humor has fascinated ocular physiologists for many years. The discrepancies in concentration of various constituents as compared with plasma levels have led to considerable speculation as to the nature of this fluid and its mode of formation. The ready availability of anterior chamber aqueous humor has led most investigators to confine their studies to this fluid. Although one finds changes in composition induced by the administration of carbonic anhydrase inhibitors, it is difficult to interpret these alterations by themselves. They may relate to variations in composition of plasma or posterior chamber aqueous humor as well as metabolic effects on ocular structures, permeability changes, or alterations in rate of aqueous flow.

The recent analyses of posterior as well as anterior chamber aqueous humor in addition to the plasma have permitted more complete explanations.[26] In addition they have made possible the development and testing of more comprehensive theories of aqueous humor dynamics and the obtaining of more valid estimates of the rate of formation of aqueous humor.[27,4] Carbonic anhydrase inhibitors have provided much needed tools for the alteration of the secretion parameter and the subsequent measurement of the changes induced in composition and rate of flow.

In a number of species the concentrations of several ions in the anterior chamber approach that of the plasma when the rate of flow is decreased by carbonic anhydrase inhibitors (for example, the bicarbonate deficit and chloride and hydrogen ion excesses in the human eye, or the bicarbonate excess and chloride and hydrogen ion deficits in the rabbit eye). However, other constituents deviate further from plasma values after acetazolamide (for example, the increase in the excess of ascorbate in the anterior chamber after acetazolamide) (table 2).

In those species where one can get closer to the secretory site by withdrawing fluids from

TABLE 2

Effect of acetazolamide administration* on the average steady-state composition of the anterior chamber aqueous humor

Ion	Chloride	Bicarbonate	Hydrogen Ion	Ascorbate	Lactate
Human					
Before	1.08	0.83	1.53	15	—
After	1.02	0.94	1.30	18	—
Rabbit					
Before	0.94	1.35	0.70	44	2.5
After	0.96	1.11	0.89	52	5.8
Guinea Pig					
Before	0.90	1.42	0.73	17	—
After	0.93	1.19	0.83	24	—

* Dose for man 500 mg. orally every six hours; rabbit 100 mg./kg. intravenously followed by 25 mg./kg. every 30 minutes; guinea pig 70 mg./kg. intraperitoneally followed by 35 mg./kg. every 60 minutes. All values are for non-nephrectomized animals and are expressed as ratio aqueous to plasma concentration in mM/kg. water.

TABLE 3

EFFECT OF ACETAZOLAMIDE ADMINISTRATION* ON THE STEADY-STATE COMPOSITION OF THE POSTERIOR CHAMBER AQUEOUS HUMOR

Ion	Chloride	Bicarbonate	Ascorbate	Lactate
Rabbit				
Before	0.88	1.70	57	3.1
After	0.94	1.39	86	7.2
Guinea Pig				
Before	—	1.52	19	—
After	—	1.30	30	—

* Dose for rabbit 100 mg./kg. intravenously followed by 25 mg./kg. every 30 minutes. Dose for guinea pig 70 mg./kg. intraperitoneally followed by 35 mg./kg. every 60 minutes. All values are for non-nephrectomized animals and are expressed as ratio aqueous to plasma concentration in mM/kg. water.

the posterior chamber, one finds greater deviations from plasma concentrations (for example, greater excesses of bicarbonate in the rabbit and guinea pig, and of chloride in the monkey and man (table 3). Furthermore, one is able to demonstrate that following carbonic anhydrase inhibition the anterior chamber concentrations approach plasma levels more uniformly when compared with posterior chamber values. For example, the actual increase in concentration in the anterior chamber of such substances as ascorbate is explained by the remarkable rise in the posterior chamber. Following partial suppression of aqueous formation, the concentration of nonmetabolized dissolved substances in the posterior chamber may rise, fall, or remain the same dependent upon the relative influence of the secretory inhibitor on the transfer of water and the particular solute into the eye. Thus, in the rabbit and guinea pig subjected to carbonic anhydrase inhibition, the posterior chamber bicarbonate concentration falls, but the ascorbate conentration rises (table 3).

Some observers[28, 29] have interpreted the rise in ascorbate concentration in the aqueous as an increased transfer of this anion. Actually the secretion of ascorbate is reduced by 26 to 27 percent following the administration of acetazolamide.[30] This is true for the rabbit at both normal and elevated plasma ascorbate levels (table 4). However, since the suppression of aqueous flow is approximately 50 percent, the posterior chamber concentration of ascorbate increases by about 50 percent. Similar calculations for the guinea pig also reveal some 25 percent decrease in ascorbate transfer into the posterior chamber after carbonic anhydrase inhibition.

The acetazolamide induced alterations in posterior chamber bicarbonate concentrations in both the rabbit and guinea pig may be interpreted as a greater suppression of bicarbonate transfer than of water. This may result from a decreased supply of the bicarbonate ion in the absence of carbonic anhydrase. On the other hand, the primary secretory concentration of bicarbonate may remain constant, and the reduction in bicarbonate concentration in the posterior chamber result from diffusional loss of bicarbonate. Since aqueous humor spends roughly twice as much time in the posterior chamber following carbonic anhydrase inhibition, the measured reduction of bicarbonate excess over plasma in the rabbit from about 16 mM. to 8.0 or 9.0 mM. suggests a bicarbonate concentration of 30 to 32 mM./kg. water above the plasma value in the aqueous humor as it enters the posterior chamber. Similarly in the guinea pig[31] the bicarbonate excesses over plasma in the posterior chamber are approximately 12 to 13 mM. and 6.0 mM. before and after acetazolamide respectively, indicating a concentration some 24 to 27 mM./kg. water higher than plasma levels for the primary secretory product. This would be consistent with a concentration of bicarbonate of about 50 to 55 mM./kg. water transferred into

TABLE 4

EFFECT OF ACETAZOLAMIDE* ON THE RATE OF TRANSFER OF ASCORBATE INTO THE RABBIT AQUEOUS HUMOR

	Posterior Chamber Concentration Ascorbate (mM/kg. H_2O)	Rate of Flow (μl/min.)	Transfer Rate (mM/min.)
Normal Plasma Ascorbate			
Untreated	1.64	3.5	5.7×10^{-6}
Acetazolamide	2.46	1.7	4.2×10^{-6}
Per cent change	+50%	−52%	−26%
Ascorbate Saturation			
Untreated	4.32	3.5	15.1×10^{-6}
Acetazolamide	6.44	1.7	11.0×10^{-6}
Per cent change	+49%	−52%	−27%

* Dose 100 mg./kg. intravenously followed by 25 mg./kg. every 30 minutes.

the posterior chamber of both species.

For such metabolic products as lactate the high ocular concentrations are believed to stem in large part from the glycolytic metabolism of the lens and cornea. Decreasing the rate of aqueous flow results in partial stagnation of aqueous and a rise in concentrations in both the anterior and posterior chambers.[32]

It becomes clear that one cannot predict alterations in composition of the posterior chamber from our present knowledge of the mechanism of the formation of the aqueous humor. It is therefore necessary to make posterior chamber measurements as well as anterior chamber and plasma determinations in order to attempt to interpret the effects of various inhibitors. Furthermore for the approximate estimation of changes in rate of aqueous flow from composition changes, it matters little as to the direction of the alterations in aqueous humor concentrations. Of much greater significance is the comparison of relative posterior chamber, anterior chamber, and plasma concentrations.

Using the formulation of Kinsey and Palm,[27,33] the alterations in steady state concentrations of non-metabolized solutes in the aqueous humor may be used as a measure of the ratio of the aqueous flow to the diffusion rates for the particular substance. Thus

$$\frac{dC_a}{dt} = k_f C_r + k_d(C_p - C_a) - k_f Ca \tag{2}$$

where

$\frac{dC_a}{dt}$ = the rate of change of concentration of the solute in the anterior chamber aqueous.

k_f = the rate of aqueous flow per minute as a fraction of the anterior chamber volume.

k_d = the rate of diffusional exchange per minute of the solute between anterior chamber and plasma (as a fraction of the anterior chamber volume).

C_p = concentration in arterial plasma in mM./kg. H_2O.

C_a = concentration in anterior chamber aqueous in mM./kg. H_2O.

C_r = concentration in posterior chamber aqueous in mM./kg. H_2O.

At steady-state $dC_a/dt = O$ and:

$$\frac{k_f}{k_d} = \frac{C_a - C_p}{C_r - C_a} \tag{3}$$

The errors involved in this method are many. The ratio k_f/k_d may vary with size of the animal, methods of handling, anesthesia, and the individual variations among animals. However, if these are standardized as much as possible, and if a substance is chosen which is readily measured and offers a relatively large concentration differences in anterior chamber, posterior chamber, and plasma, valid estimates of the ratio and its alteration can be made. Thus ascorbate is quite suitable for the rabbit eye because of the very different content of plasma and posterior chamber and anterior chamber

aqueous humors. On the other hand the maximum changes one might anticipate in steady state sodium distribution (one or two percent) might well be lost in the error of measurement. The steady-state distribution method has the advantage of obtaining a relative estimate on the same animal before and after the administration of an agent. However, it does assume that the two eyes of the same animal are identical. A most important limitation of the method is that it measures the ratio k_f/k_d. In order to estimate the rate of flow, assumptions or determinations must be made of the effects induced on the rate of diffusional exchange.

Some results of applying the steady state distribution method to the effects of several carbonic anhydrase inhibitors are presented in Table 5. The data summarized are limited to non-nephrectomized rabbits and guinea pigs using only topical anesthesia. Assuming no change in the coefficient of diffusion, k_d, the findings are consistent with an average decrease in rate of aqueous flow of 50 to 60 percent produced by acetazolamide in rabbits and guinea pigs, and by dichlorphenamide and methazolamide in rabbit eyes. Chlorothiazide does not produce a significant change in bicarbonate concentrations of the rabbit eye. The inactive tertiary-butyl derivative of methazolamide also fails to alter the bicarbonate ratios in significant fashion.

TABLE 5

EFFECT OF ADMINISTRATION OF CARBONIC ANHYDRASE INHIBITORS ON THE RATIO K_f/K_d

Carbonic Anhydrase Inhibitor	Rabbit		Guinea Pig	
	No. Eyes	$\Delta\frac{k_f}{k_d}$	No. Eyes	$\Delta\frac{k_f}{k_d}$
A. *Steady-state bicarbonate data*				
Acetazolamide[b]	46	−60%	20	−58%
Dichlorphenamide[a]	11	−51%	—	—
Methazolamide[a]	22	−56%	—	—
Chlorothiazide[a]	14	+15%	—	—
*CL 17, 262[a]	8	+11%	—	—
B. *Steady-state ascorbate data*				
Acetazolamide[b]	60	−55%	16	−53%

[*] Tertiary butyl derivative of methazolamide (inactive as carbonic anhydrase inhibitor).

[a] 20 mg./kg. intravenously.

[b] Rabbit dose 100 mg./kg. intravenously followed by 25 mg./kg. every 30 minutes. Guinea pig dose 70 mg./kg. intraperitoneally followed by 35 mg./kg every 60 minutes. (All non-nephrectomized animals.)

IV. THE EFFECT OF CARBONIC ANHYDRASE INHIBITORS ON THE TURNOVER OF TEST SUBSTANCES IN THE AQUEOUS HUMOR

Curves representing the rate of accumulation of a systemically injected test substance in the aqueous humor can be obtained by the use of a large number of animals.[33] If the blood level is kept constant and posterior and anterior chamber measurements are made only once for each eye, the data at various time intervals may be interpreted so as to obtain estimates of rate of aqueous flow.

From equation (2) it is apparent that:

$$\frac{dC_a}{dt}\Big/(C_p - C_a) = k_f\frac{(C_r - C_a)}{(C_p - C_a)} + k_d. \tag{4}$$

This is an equation of the form $Y = k_fX + k_d$ where:

$$Y = \frac{dC_a}{dt}\Big/(C_p - C_a)$$

$$X = \frac{C_r - C_a}{C_p - C_a}$$

The turnover data for posterior chamber, anterior chamber, and plasma furnish estimates of Y and X for different time intervals after the injection. Plotting Y vs. X results in a straight line with slope k_f and Y-intercept k_d.[4] One may then compare turnover data on a simultaneously studied series of animals subjected to carbonic anhydrase inhibitors and determine the alteration in k_f.

The errors involved in the turnover method are related to the assumptions made in equation (2), as well as those in similar formulations for posterior chamber and vitreous dynamics.[4,35] In addition, the disregard of, or assumptions about, lens and corneal exchange may make the absolute values inaccurate. Variations in individual animals, techniques of sampling and handling, anes-

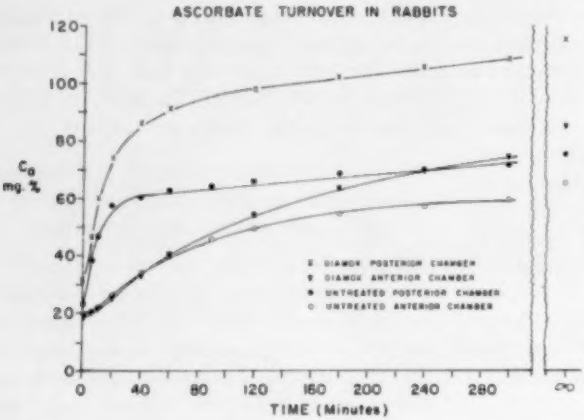


Fig. 3 (Becker). Mean concentrations of ascorbate in the posterior and anterior chamber aqueous humors following intravenous ascorbate in both untreated (circles) and acetazolamide-treated (x and ▼) rabbits (reference 36).

thesia, and so forth, may result in large errors. Again the relative alterations induced by carbonic anhydrase inhibition are more valid than the absolute measurements.

Ascorbate is a particularly useful substance for this type of study. It has the advantages of being easily measured and of entering the eye almost entirely by secretion.[27] Furthermore, the limited secretory capacity for ascorbate makes the blood level less critical as long as it is above that which results in maximum secretory transfer.

In Figure 3 are plotted the curves for the rate for accumulation of ascorbate in the aqueous humor of the posterior and anterior chambers of untreated and acetazolamide-treated rabbits.[36] Acetazolamide alters the steady-state concentration of ascorbate and the curves therefore differ considerably. It is to be noted, however, that if only anterior chamber concentrations are considered, no significant differences in concentration are noted between the two groups of animals at times earlier than two hours. By X-Y analyses, these data are interpreted as representing approximately a 50 percent decrease in rate of aqueous flow induced by acetazolamide. No changes are noted in the diffusion constant for ascorbate following acetazolamide (fig. 4 and table 6). The anterior chamber data may also be analyzed by the exponential method of Friedenwald.[4] Values for k_{out} ($k_d + k_f$) for the anterior chamber obtained in this fashion are compatible with the X-Y data and a 50 percent suppression of secretion. Obviously it would be fallacious to conclude that no changes in flow rate occur on the basis of incomplete anterior chamber data obtained at time intervals up to two hours (fig. 3).

When turnover of radioactive sodium is studied in this fashion, one is handicapped additionally by the large fraction of this substance which enters the eye by diffusion as

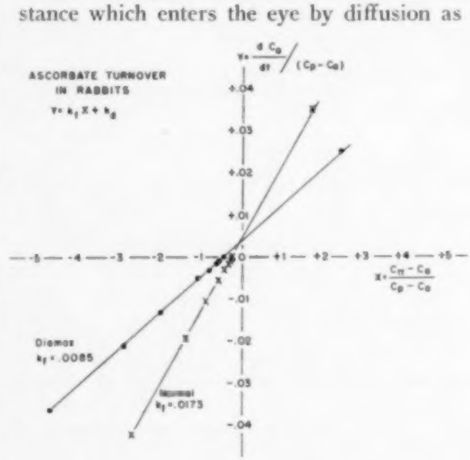


Fig. 4 (Becker). Linear (X-Y) analysis of ascorbate turnover in acetazolamide-treated (circles) and untreated (-X-X-) rabbits. The slopes of the straight line provide estimates for k_f, the flow coefficients.

TABLE 6

ANALYSIS OF TURNOVER DATA OF RADIOACTIVE SODIUM AND ASCORBATE IN UNTREATED AND ACETAZOLAMIDE TREATED RABBITS

	Normal		Acetazolamide	
	Na	AA	Na	AA
Linear $(X-Y)$				
100 k_f	1.6%	1.7%	0.7%	0.9%
100 k_d	1.2%	0.5%	1.2%	0.4%
*Exponential**				
100 k_a	2.7%	2.2%	1.9%	1.3%
100 k_1	9%	10%	6%	7%

k_f = the rate of aqueous flow per minute as a fraction of the anterior chamber volume.
k_d = rate of diffusional exchange of the solute between anterior chamber and plasma.
k_a = the rate of transfer of the test substance out of the anterior chamber as a fraction of that chamber (corrected for the turnover rate of the reservoir k_2); $k_a = k_d + k_f$.
k_1 = the rate of transfer of the test substance out of the posterior chamber as a fraction of that chamber (corrected for the reservoir factor k_2).
* k_2 (reservoir filling factor) for sodium 24 = 0.0042.

well as by the failure of carbonic anhydrase inhibition to alter the steady-state concentration significantly. In preliminary unpublished data obtained with Friedenwald in 1953, only small differences were noted in the turnover of radioactive sodium between normal and acetazolamide-treated two-kg. rabbits. The average data for four to six eyes at various time intervals are plotted in Figure 5. It will be seen that the acetazolamide-treated animals demonstrate a very small lag in the rate of accumulation of sodium in both anterior and posterior chambers. However, the differences may be within the experimental error. These findings are in excellent agreement with those reported by other observers. [37, 38] Although the number of eyes at each point are inadequate, the data are presented here merely to indicate again that one cannot conclude from the similarities of the *anterior chamber curves alone* that there has been no influence of acetazolamide on rate of aqueous flow. As a matter of fact, the X-Y and exponential analyses of the average *posterior and anterior chamber turnover data* as plotted reveal values of k_f for normal rabbits in excellent agreement with those resulting from Kinsey and Palms' data as well as the above ascorbate data.[27, 36] Furthermore, after acetazolamide the flow rate is decreased and may be interpreted as some 50-percent inhibition of aqueous formation by both X-Y and exponential analysis (figs. 6, 7, and 8 and table 6). It is apparent that the turnover of sodium 24 is not a very sensitive measure of rate of aqueous flow, and becomes even less reliable if the anterior chamber alone is considered at a few time intervals.

The appearance time of fluorescein through the pupil into the anterior chamber after its intravenous administration is a crude shortcut for relative estimates of rate of aqueous flow. By measuring the time interval between the appearance of fluorescein

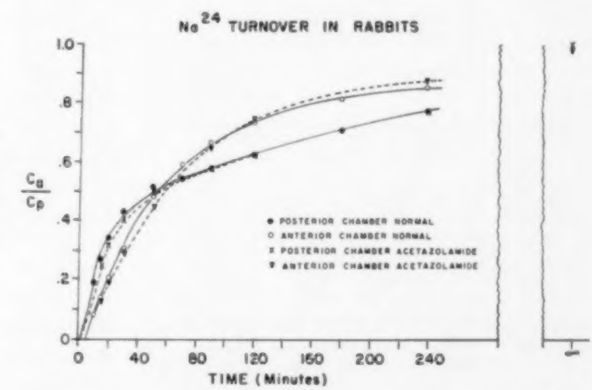


Fig. 5 (Becker). Average relative concentrations of radioactive sodium in the posterior and anterior chamber aqueous humors following intraperitoneal injections of sodium 24 in both untreated (circles) and acetazolamide-treated (x and ▼) rabbits.

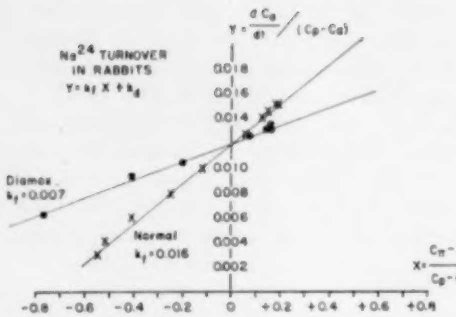


Fig. 6 (Becker). Linear (X-Y) analysis of sodium turnover in acetazolamide-treated (circles) and untreated (-X-X-) rabbits. The slopes of the lines provide estimates for k_f, the flow coefficients.

in the conjunctival vessels and through the pupil, a value is obtained that is related to

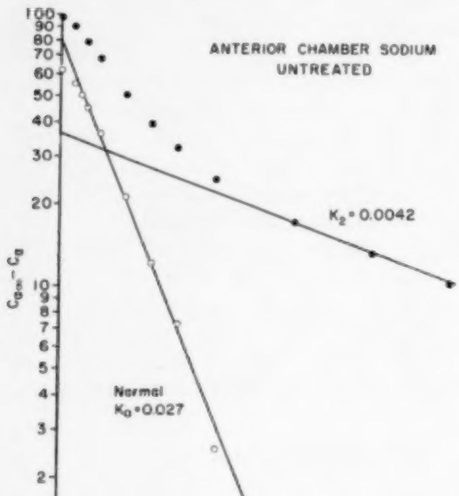


Fig. 7 (Becker). Graphic exponential analysis of the anterior chamber radioactive sodium data for untreated rabbits (fig. 5). The ordinate plotted (solid circles) is the log ($C_{a\infty} - C_a$) where $C_{a\infty}$ is taken as 98 percent of plasma concentration. Similar analysis of the untreated posterior chamber data ($C_{\pi\infty} = 99$ percent) revealed a linear tail of slope $k_2 = 0.0042$. Subtracting this line from the anterior chamber data results in a straight line (open circles) with slope k_0 ($= k_f + k_d$) $= 0.027$.

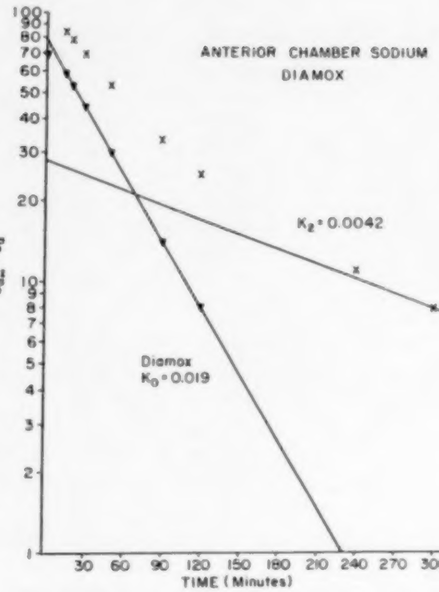


Fig. 8 (Becker). Graphic exponential analysis of the anterior chamber radioactive sodium data for acetazolamide-treated rabbits (fig. 5). The ordinate plotted (-X-X-) is the log ($C_{a\infty} - C_a$) where $C_{a\infty}$ is taken as 98 percent of plasma concentration. Similar analysis of the acetazolamide-treated posterior chamber data ($C_{\pi\infty} = 99$ percent) revealed a linear tail of slope $k_2 = 0.0042$. Subtracting this line from the anterior. chamber data results in a straight line (-▼-▼-) with slope k_0 ($= k_f + k_d$) $= 0.019$.

the transit time from ciliary process to anterior chamber.[39, 40] This time interval varies greatly among eyes, and has no absolute significance. However repeated determinations on the same eye are quite reproducible if care is taken to avoid variations in technique and especially alterations in permeability. The method has the advantage of simplicity, applicability to human as well as animal eyes, the opportunity to compare it with other methods (for example, tonography) on the same eye, and the possibility of repeat measurements after attempts to alter aqueous flow. The delay in fluorescein appearance time that follows acetazolamide administration in carefully studied patients and rabbits suggests some 50 to 55-percent decrease in rate of aqueous flow in both species[39, 41] (table 7).

TABLE 7

THE EFFECT OF ACETAZOLAMIDE ADMINISTRATION ON THE APPEARANCE TIME[a] OF FLUORESCEIN

Species	No. Eyes	Untreated Time (T_1)	Acetazolamide[b] Time (T_2)	$\Delta F\%$
Rabbit	22	63	124	−49%
Human	16	95	205	−54%

[a] Mean value in seconds.
[b] 100 mg./kg. intravenously for rabbits; 500 mg. orally for man.

$$\Delta F\% = 100\left[\frac{T_1 - T_2}{T_2}\right]$$

V. Summary of the effects of various carbonic anhydrase inhibitors on rate of aqueous formation

It is most reassuring that such diverse approaches to the problem of estimating alterations in the rate of secretion provide data consistent with a 50 to 60-percent decrease in flow in rabbit eyes by a number of potent carbonic anhydrase inhibitors (tables 1, 5, 6, and 7). The agreement obtained by such completely independent methods of measurements, each with its own assumptions and errors, strongly suggests the basic validity of current concepts of aqueous humor dynamics and methods of measurement of rate of formation of aqueous humor. It is also of interest that even with massive doses of various different effective carbonic anhydrase inhibitors, some 40 to 50 percent of the normal rate of secretion persists. This may be attributed to the adequacy of the uncatalyzed rate of the hydration and dehydration of carbon dioxide for the residual secretion, or to aspects of the secretory process that are independent of the enzyme carbonic anhydrase. Although providing a limitation on the therapeutic efficacy of carbonic anhydrase inhibitors, the remaining flow may well explain the absence of ocular toxicity and be the safety factor avoiding the consequences of inadequate nutritional supply to the lens and cornea.

VI. The site of action of carbonic anhydrase inhibitors

Although the question of the site of action has not been entirely resolved, there is evidence that these agents act directly on the ciliary processes. This is supported by the finding of carbonic anhydrase in the ciliary processes[42] and its inhibition by acetazolamide.[43, 44] In addition, the rapidity of the pressure fall after intravenous administration in all species studied suggests a direct effect upon the eye. That the effect on the eye is independent of renal effects has been established by the use of nephrectomized animals and by blocking the renal effect by preadministration of ammonium chloride.[45] Furthermore, the use of carbonic anhydrase inhibitors such as methazolamide in doses that alter renal function minimally, results in decreases in intraocular pressure and aqueous flow which are of similar magnitude to those following more marked generalized carbonic anhydrase inhibition. Thus, the administration of 100 mg. of methazolamide is associated with very little change in plasma electrolytes or pH in humans, but causes similar relative changes in composition and rate of aqueous formation to those that follow 500 mg. of acetazolamide[46] (table 8). In addition, species such as the elasmobranch which have no carbonic anhydrase in their kidneys and therefore demonstrate no renal effect or alterations in plasma bicarbonate concentrations, still obtain a fall in aqueous bicarbonate after acetazolamide administration.[47] More conclusive evidence for a direct effect on the eye stems from the carotid injection experiments of Wistrand.[10] When isotonic acetazolamide is injected into one carotid of a cat in small doses and an inactive substituted sulfonamide in the other, the fall in intraocular pressure is more rapid and much more pronounced in the eye homolateral to the acetazolamide infusion.

Although it may be concluded that carbonic anhydrase inhibitors act directly upon the ciliary processes, this does not rule out

TABLE 8

COMPARISON OF AVERAGE EFFECTS OF ACETAZOLAMIDE AND METHAZOLAMIDE ON HUMAN EYES

Anterior Chamber Chemistries[a]	Normal	Methazolamide	Acetazolamide
No. Eyes	22	12	18
Chloride	1.08	1.02	1.02
Bicarbonate	0.83	0.93	0.94
Hydrogen ion	1.53	1.29	1.30
Ascorbate	15	20	18
Plasma values			
pH	7.40	7.39	7.35
Bicarbonate[b]	25.9	23.1	20.5
Tonographic alteration in rate of flow			
No. Eyes	—	64	126
ΔF	—	−46%	−52%

Dose of acetazolamide 500 mg. every six hours; methazolamide 100 mg. every eight hours orally.
[a] Expressed as the ratio of concentration in aqueous humor to that in plasma water.
[b] Concentration in mM/kg. water.

possible systemic alterations as contributing to the pressure fall. There can be no doubt that the renal effects of such agents as acetazolamide alter plasma pH and electrolytes. Furthermore, one might anticipate variations in the buffering capacity of the ciliary epithelial cell with marked changes in circulating electrolytes. The role of such side-effects in the clinical therapy of glaucoma remains unknown. There is evidence that in some patients acidosis will potentiate the fall in intraocular pressure that follows acetazolamide, but acidosis itself has little effect unless very severe and carbonic anhydrase inhibitors can lower intraocular pressure without systemic acidosis. The problem of failure of carbonic anhydrase inhibitors to act topically on the eye (even in the presence of systemic acidosis) is unfortunate clinically, and remains unexplained except for speculation as to penetration of the drug in adequate concentration to the active enzyme site in vivo.[45, 48–50]

VII. MODE OF ACTION OF CARBONIC ANHYDRASE INHIBITORS

A. THE ROLE OF CARBONIC ANHYDRASE IN SECRETORY PROCESSES

Carbonic anhydrase has been demonstrated to be present and to play an active role in a number of secretory processes. Most of these involve the formation of a product which differs in hydrogen ion concentration from plasma. Thus, the gastric mucosa,[51–53] the cerebrospinal fluid,[54, 55] and the primate eye transfer an acid secretion; whereas, the pancreas[58] and the rabbit or guinea pig eye secrete an alkaline product. All of these transport processes are suppressed in part when carbonic anhydrase is inhibited.[56, 57] The mammalian renal tubule secretes hydrogen ions and reabsorbs bicarbonate;[59] the alligator renal tubule secrets bicarbonate and reabsorbs chlorides.[60] Carbonic anhydrase inhibition results in increased bicarbonate loss in the urine by mammals and systemic acidosis; but it increases the chloride output in alligators resulting in systemic alkalemia. The fresh water fish with a plasma chloride in large excess over surroundings demonstrates a fall in plasma chloride after acetazolamide; but the salt water fish with a deficit of chloride compared to his surroundings experiences a rise in plasma chloride concentration when subjected to carbonic anhydrase inhibition.[61]

In each of these sites a number of theories as to the secretory mechanism and the role of carbonic anhydrase have been postulated. Most theories fall into two categories, attributing either a direct or an indirect role to the enzyme. Those who believe carbonic anhydrase to enter *directly* into secretory process consider it as a means of providing the H^+ or HCO^-_3 needed for the secretory process from the carbon dioxide of local metabolic processes or by diffusion from the blood. These ions may then be exchanged for the cations or anions to be reabsorbed, secreted, or transported.[62] On the other hand the H^+ or HCO^-_3 may be transferred or extruded from the cell by specific ion carriers. The advocates of an *indirect* role of carbonic anhydrase believe it to provide buffering capacity for the cell.[63] Thus cells secreting acid will themselves become alkaline and need a supply of hydrogen ions to maintain a pH permitting continued secretory function. Other cells secreting fluids of high pH or

exchanging sodium for hydrogen ions, accumulate hydrogen ions and need a supply of bicarbonate to avoid excessive fall in pH. The nature and degree of the secretory suppression induced by carbonic anhydrase inhibitors will depend upon which link or links in the secretory process are most susceptible to the altered pH of the cell. With the ubiquitous carbon dioxide produced locally or diffusing readily from the blood stream, carbonic anhydrase is said by Davenport[64] to provide a "clean-up mechanism which gets tissues out of the trouble they have got themselves into by secreting anions or cations." The effectiveness of carbonic anhydrase inhibitors will depend on their penetration to the enzyme site and the needs of the secretory cell for obtaining or ridding itself of the products of this reversible reaction.

B. Carbonic anhydrase and the formation of aqueous humor

Historically it was the redox pump mechanism,[65,66] as modified for the rabbit eye by Friedenwald,[1] which first suggested a role for carbonic anhydrase in the secretory mechanism (fig. 9). In this type of hypothesis electrons are transferred from stroma to epithelium with the formation of hydroxyl ions. Carbonic anhydrase plays the *indirect role* of providing buffering capacity for both the postulated residual hydrogen ions in the stroma and the hydroxyl ions produced in the epithelium.[67] Thus a high concentration of bicarbonate and an alkaline pH in the aqueous humor were anticipated by Friedenwald. The experimental confirmation of these predictions in rabbit eyes[26] and the finding of carbonic anhydrase in the rabbit ciliary epithelium[42] led to the experimental trial of carbonic anhydrase inhibitors. In the primate eye, as in the gastric mucosa, such a pump would have to transfer electrons in the opposite direction with the production of hydrogen ions in the posterior chamber. This could be followed by an ion exchange of sodium for hydrogen as postulated for the renal tubule.[68]

The redox pump mechanism does not appear to satisfy the rate of transfer of ions into the aqueous stoichiometrically, that is, the oxygen consumption of the ciliary body is not high enough to account for the postulated number of electrons transferred.[69] This has proved equally true for similar postulates about the frog skin, stomach, and kidney.[70] However, with suitable modifications some of these objections can be avoided One may postulate the secretion of only the excess over plasma of a given ion into the aqueous humor. This would be a much more economical process. The recycling of electrons by carriers that can be reduced and phosphorylated such as postulated by Davies provides another solution.[71] In such schemes hydrogen ions are derived from water (or carbonic acid) instead of from the oxidation of RH_2, and the limitations imposed by oxygen consumption are avoided. Energy is obtained by coupling the ion transport to oxidative phosphorylation and there is no net movement of charge.

The *direct* role of carbonic anhydrase may be postulated to provide bicarbonate or hydrogen ions for the secretory process. This might take place by means of a suitable bicarbonate carrier, and the residual hydrogen ions in the cell reabsorbed by forced exchange with sodium from the plasma (fig.

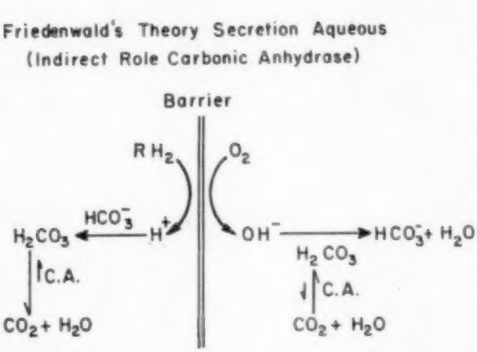


Fig. 9 (Becker). Postulated redox-pump mechanism for the formation of aqueous humor in the rabbit. Carbonic anhydrase (C.A.) provides buffering capacity for the residual hydrogen ions in the stroma and the hydroxyl ions produced in the epithelium.

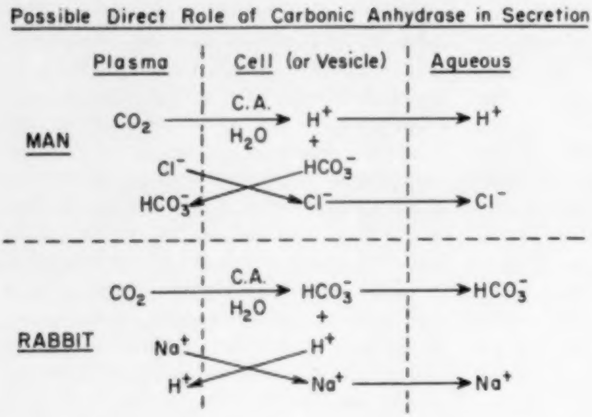


Fig. 10 (Becker). Carbonic anhydrase as a means of providing directly from carbon dioxide and water, the hydrogen ions or bicarbonate ions for transfer into the aqueous humor. The residual ion may be reabsorbed by forced exchange.

10). On the other hand, the exchange of hydrogen ion of the ciliary epithelium for sodium of the blood might be the primary process, and the resulting sodium bicarbonate of the cell transferred into the posterior chamber. This is analogous to the transport of sodium and acidification of urine in the renal tubule.[72] Carbonic anhydrase can play either the direct role of providing the hydrogen ions, or the indirect role of buffering the residual hydroxyl ions in the cell if hydrogen is derived from water. Either process could explain the high bicarbonate and low hydrogen ion concentration as well as the sodium excess in the aqueous humor of the rabbit eye.

In the primate eye, however, the composition would suggest an exchange in the reverse direction. Chloride might be transported into the eye by a specific carrier or by forced exchange with bicarbonate, the residual hydrogen ions subsequently entering the eye and exchanging in part with sodium. This would account for the low pH and high chloride content of the human aqueous humor (fig. 10). One can even devise multiple transport mechanisms, or couple the transfer of a cation and anion, recycling electrons, and deriving the energy from oxidative phosphorylation (fig. 11). This source of energy becomes especially attractive as contributing to the secretion of aqueous humor because of its activity in ciliary processes.[73] Furthermore the intracarotid administration of styryl quinoline dyes, which concentrate in the ciliary epithelium and uncouple oxidative phosphorylation, appear to result in a prolonged but reversible suppression of aqueous humor formation.[74]

The recent suggestion of membrane flow and pinocytosis as mechanisms for ion transport[75] has revived considerable interest in the phenomenon of "cell-drinking."[76] Pease[77] found an elaborate deep infolding of the surface of the ciliary epithelium of the rat, and rows of droplets continuing from the depths of the folds. Holmberg[9] elaborated on these electron microscopic studies and noted that the small cytoplasmic vesicles in the rabbit ciliary epithelium accumulated within the cell when secretion was partially suppressed by carbonic anhydrase inhibition. These observations raise the question as to the possible role of the uptake of droplets of environmental fluid (pinocytosis) in the formation of aqueous humor. One can postulate the pinching off of a blood dialysate into the vesicle and its modification by the cell during the transport of the vacuole to its site of emptying into the posterior chamber.

Recently doubt has been expressed as to the role of hypertonicity of aqueous humor as a driving force for the transfer of water and maintenance of intraocular pressure.[78-80] The question has been raised because of the failure to alter the apparent osmotic excess

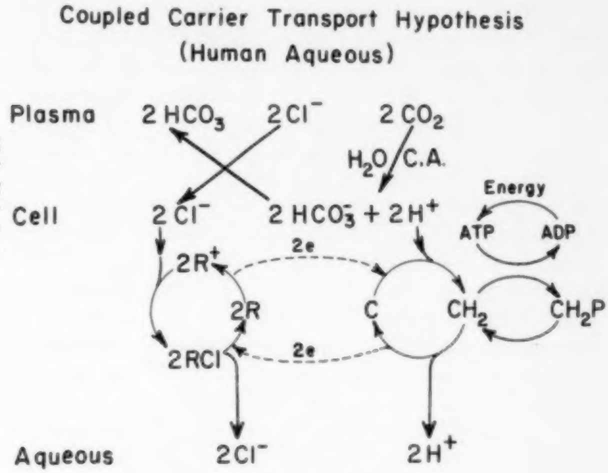


Fig. 11 (Becker). Hypothetical mechanism for the coupled transfer of a cation and anion deriving energy from oxidative phosphorylation. The example given is as in Figure 10 for human aqueous humor.

in rabbit aqueous humor in spite of effective lowering of intraocular pressure by acetazolamide. Furthermore, experimental alterations of osmotic factors fail to alter aqueous flow as much as anticipated.[78] This may mean that the measured hypertonicity merely results from water loss by evaporation through the cornea[1] or perhaps from the errors of in vitro methods used for the determination.[80] Such doubts as to the validity of an osmotic excess in the aqueous humor as compared to the plasma in iris vessels in vivo tend to support the pinocytosis hypothesis.

If Friedenwald's hypothesis for the rabbit is to be maintained and modified, only the excess of hydroxyl ions would have to be formed by electron transport into the vacuole. This might amount to only 0.05 to 0.10μM per minute, requiring only 0.25 to 0.5 microliters of oxygen per minute or one fourth or one half the measured respiration of rabbit ciliary processes.[69] On the other hand, any other secretory mechanism can be postulated as taking place into the vesicle instead of directly into the posterior chamber. Carbonic anhydrase could still play a direct or indirect role in the formation of this vacuolar secretion. In addition, carbonic anhydrase might be necessary to maintain the rate of transfer of vacuoles across the cell. Following carbonic anhydrase inhibition, the slower passage of vesicles would increase the number seen in electron micrographs.[9] The reduced rate of vesicle transport could result from alterations in cell pH, decrease in supply of a substrate or secreted ion, or from a more direct interference with the poorly understood and possible ATP linked-energetics of vacuole transport. The observed increase in width of mitochondria of the ciliary epithelium which follows acetazolamide administration is of interest in this regard.[9, 81] Further electron microscopic studies are needed in order to learn the effects of altered pH, uncouplers of oxidative phosphorylation, other ocular hypotensive agents, and the lowering of intraocular pressure itself on the appearance of vesicles in the ciliary epithelium.

An entertaining theory to explain the behavior of ascorbate following carbonic anhydrase inhibition stems from the pinocytosis concept. Since the concentration of ascorbate in the ciliary epithelium is extremely high, one may postulate its diffusion into the vacuole as it passes through the cell. If the vacuole is then slowed down in its movement by some 50 percent after acetazolamide, the additional time provided for diffusional exchange could account for the 50-percent rise induced in posterior chamber concentration

of ascorbate. For example, if the ascorbate concentration in the rabbit ciliary epithelium is 3.28 mM./kg. water and the normal time spent by the vacuole in the cell results in the average value of 1.64 mM./kg. water found in the normal posterior chamber, doubling the transit time of the vacuole results in a rise to the average of 2.46 mM./kg. water reported in the acetazolamide-treated rabbits. Similarly, in the ascorbate-saturated rabbit a concentration in the cell of 8.5 mM./kg. water would account for the 4.2 mM./kg. water in the posterior chamber before acetazolamide and the 6.4 mM./kg. water after carbonic anhydrase inhibition. The posterior chamber ascorbate data for guinea pig eyes may be interpreted in the same fashion.

The knowledge that carbonic anhydrase is necessary for the formation of normal amounts of aqueous humor has not resolved the details of the mechanism of aqueous secretion. In fact, it has raised more questions than it has answered. The unsolved problems of ion transport, energetics, and mechanisms of fluid formation are shared with a large group of workers interested in secretory problems in the renal tubule, gastric mucosa, choroid plexus, red cells, lens, frog skin, and even the individual cell. In spite of an enormous amount of work and an extensive literature, the details of each of these processes remains speculative and obscure. However, no matter what the intimate mechanism, the ability to alter the rate of formation of aqueous humor has been of enormous help in putting to the test available methods and theories of the measurement of rate of aqueous flow. It is certain that other agents will be found to alter the secretory process. In addition to their use in the therapy of glaucoma, it is hoped that these will prove of as great heuristic value as acetazolamide in the study of the rate of aqueous flow, as well as providing us with greater insight and understanding of the intimate nature of the mechanism of aqueous humor formation.

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PROTEIN DYNAMICS IN THE EYE STUDIED WITH LABELLED PROTEINS*

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The intraocular fluids contain a very small concentration of soluble proteins which appear to be identical with the proteins of the plasma. Little is known about the mechanism whereby they enter and leave the eye or to what extent they may exchange between its chambers. Owing to the minute quantities available in the intraocular fluids, the study of the dynamics of their transfer by introducing radioactive proteins in the blood requires inconveniently high levels of activity. It was decided in the first place, therefore, to investigate the movements of a protein, radioactive-iodine labelled albumin, after its injection into the vitreous body. I have shown previously[8] that, by applying this technique to the radioactive sodium ion, its movement between the chambers of the eye appeared to be limited only by rate of free diffusion of the ion in the vitreous body. The direct exchange between the vitreous humor and the blood was, however, restricted by a membrane of low permeability, probably the retina.

Methods

Albino rabbits were used as the experimental animals, and, to reduce the risk of a reaction in the eye, rabbit albumin was used as to the test material. This was prepared by labelling separated albumin or whole serum with I^{131} by the method of Margen and Tarver;[7] on the average, one atom of I was incorporated in each molecule of protein. The material initially had an activity of about 50 mc./gm. A volume of five to 10 µl. of two to 10-percent solution was injected through a fine needle (30 gauge) into the vitreous body, as described previously,[8] with the animal under pentothal anesthesia. The animal's other eye was untouched.

The total activity in the eye at any time was measured by holding the head of the animal with its eye in close proximity to the sensitive area of a scintillation counter. Readings were taken in triplicate or more, in order to reduce the error to less than five percent. A standard source of I^{131} was used as a comparison on each occasion to compensate for radioactive decay and changes in counter sensitivity.

After various periods of time, the animals were killed and the aqueous humor immediately collected by paracentesis. The eye was then enucleated and the lens and vitreous body dissected out through a cut in the sclera. The vitreous body was cut free of the lens and its structure broken in a syringe. The activity of equal volumes of the two fluids was compared with a scintillation counter. Generally, the protein in these samples was

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precipitated with 10-percent trichloroacetic acid after the addition of a little inert serum. The precipitates were centrifuged, washed, and again counted. In some cases the samples were subjected to electrophoresis, the distribution of radioactivity in the strips determined with a scanner, and the area under the albumin peaks compared.

Electrophoresis

In preliminary trials electrophoresis in an agar block was found to have two advantages over that on paper. First, the entire volume of the aqueous humor can be treated without previous concentration or dialysis; second, there is negligible trailing of the albumin spot.

The technique employed was essentially that of Bussard and Perrin[3] but the use of any cooling arrangement was obviated by reducing the thickness of the block and the concentration of the buffer. It was found most convenient to use 0.03 M phosphate buffer at pH 7.0 in 0.75-percent agar for the plate. The agar solution was filtered while hot but not otherwise purified. The sample of aqueous or vitreous humor was warmed to 40°C. and mixed with one-half its volume of 2.25-percent agar at the same temperature. The mixture was then quickly transferred to a rectangular mould consisting of two L-shaped plastic strips two-mm. thick, lying five-mm. apart on a greased plate and allowed to set. The formed origin was then slid into position on a glass sheet coated with 2.25-percent agar; the aqueous and vitreous samples from one eye were run side by side. The glass sheet was then levelled and the agar forming the block was poured on a 60°C. to a depth of two mm. The electrophoresis was run overnight and in the morning the block was fixed in 10-percent trichloroacetic acid, washed, dried in hot air, and stained with bromphenol blue. While in the fixing bath, the block was transferred from the glass on to a length of cellophane tubing stretched over a former. The dried strip was cut free and fed through a radioactive scanner.*

Results

Reaction of eye

The eyes were examined with ophthalmoscope and slitlamp before and at various intervals after the injection. The typical reaction was the formation of slight punctate opacities in the vitreous body which appeared after a few days and seemed to settle on the posterior lens surface whence they were gradually absorbed. There was frequently also a faintly discernible increase in the intensity of the aqueous flare which returned to normal within five to six days. The fundus remained clear and there were no signs of inflammation in the anterior segment. Grossly, the vitreous appeared as solid as on the control side. Many eyes showed no visible reaction to the injection and a number of others showed a more marked one with a thick aqueous flare. In general, these also cleared up within a few days.

A few animals had tonometric measurements made on both eyes before the injection and tonometric and tonographic measurements at various periods after. No significant differences were found between the two eyes within a few days after the injection.

Rate of loss of activity

In all the eyes tested for a sufficient period the total activity as measured with an external counter ultimately showed an exponential decrease with time. In some eyes this decline could be followed for up to four weeks without any change of slope. In the first few days, however, the activity generally appeared to remain constant or even to rise (fig. 1). This behavior has already been found[8, 2] in the case of Na^{24} and Cr^{51} tagged hemoglobin and has been ascribed to the forward diffusion of the active material through the vitreous body from its point of injection. Attempts to correlate the rise or fall of the curve with the point of injection of the albumin or the extent of the inflammatory response were unsuccessful.

* Nuclear Chicago Model C-100A.

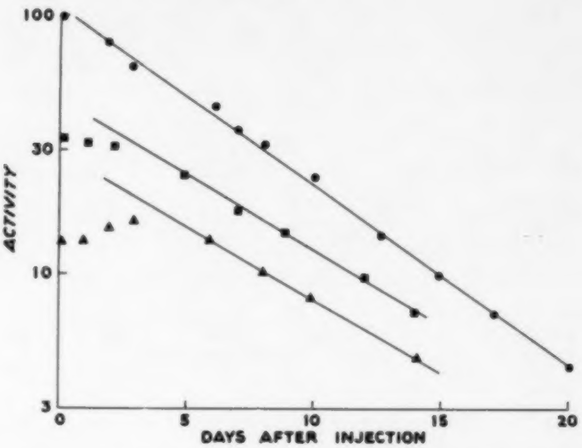

Fig. 1 (Maurice). Rate of loss of activity from eye. Points from three animals showing various behavior in early stages. Ordinate represents counts obtained from eye divided by counts from standard source of I^{131} in arbitrary units.

The rate of loss of active material from the eye could be measured from the slope of the curve. Values were accepted only from 19 experiments which lasted at least seven days and had at least four points conforming to a straight line. Of these, three aberrant values were rejected. The remaining 16 curves had a mean decay constant of 0.153/day with a standard deviation of 0.019/day.

In the experiments[8] with Na^{24} the activity of the eye on the noninjected side was counted and its value was subtracted from that of the experimental eye. This corrected for the activity which accumulates in the body of the animal and for any re-entry from the blood into the eye. This precaution was taken in some of the experiments with the protein, but, because it seemed to have no influence on the slope of the curve and the re-entry into the eyes was found to be negligible, it was not always observed. A more careful examination shows that a small increase in the estimate of the slope of the curve does, in fact, result. This amounted to seven percent on the average in seven eyes, and it was thought that the estimate derived from the 16 eyes should be corrected by the same factor and hence raised to 0.163/day.

Electrophoresis

Stained agar electrophoresis strips prepared from the original tagged material showed good resolution of the albumin from the globulins. Most of the radioactivity was found at the albumin peak; about 20 percent of it trailed behind, however, but showed no concentration in the region of the globulin bands. On electrophoresing the material in the albumin band a second time it was found to be free of any trailing activity. There was little difference in the radioactive electrophoresis pattern of the labelled albumin or whole serum.

The aqueous humor in these experiments was not concentrated, with the result that only the albumin band could be revealed by staining. A weaker albumin band could also be seen in the vitreous humor electrophoresis strips and, in addition, some stained material remained at the origin. The radioactivity of the intraocular fluid strips showed the same distribution as for the serum; the albumin peaks had the same mobility, but appeared to be a little broader.

About five percent of activity of the original tagged protein, after dialysis, was not precipitated by trichloroacetic acid. Virtually all the activity in the intraocular fluids was precipitated.

Aqueous humor concentration

The ratio of the radioactivity of the aqueous humor to an equal volume of the vitreous

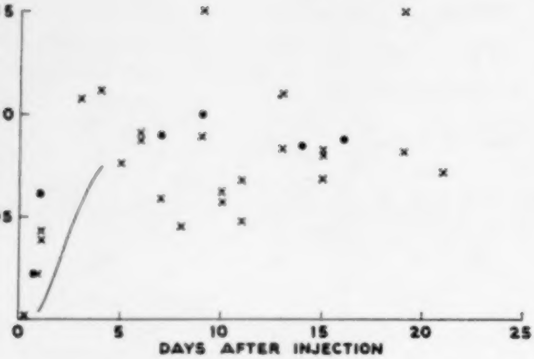

Fig. 2 (Maurice). Ratio of activities in unit volumes of aqueous humor and vitreous humor. Each point obtained from one animal. Crosses correspond to total activity; circles to activity in albumin electrophoresis band. Curve obtained from thermal analogue as described in text.

body is plotted in Figure 2. After a delay of about 12 hours before any active material enters the anterior chamber, the ratio rises rapidly and appears to level off at an average value of between 0.07 and 0.08.

Discussion

Vitreous-aqueous exchange

The first point to be examined is what proportion of the labelled albumin leaves by way of the anterior chamber. This is readily determined from the rate of outflow of the aqueous humor and the ratio of the activity in the anterior chamber to that in the vitreous body. The outflow from the anterior chamber is $k_f V_a$ where k_f is the outflow coefficient and V_a the volume of the anterior chamber. Active material is therefore lost from the anterior chamber at a rate $k_f V_a C_a$, where C_a is the activity of unit volume of aqueous humor. Since C_a varies very slowly with time, this is also the rate at which activity is lost from vitreous to the aqueous humor. The total activity in the vitreous body is given by V_vC_v and so this rate of loss corresponds to a transfer coefficient out of the vitreous body, k_{va}, given by

$$k_{va} = k_f \frac{V_aC_a}{V_vC_v}$$

The ratio V_a/V_v is 0.17.[8] C_a/C_v has been found in this paper to be about 0.07 at equilibrium and estimates of k_f vary from 0.011 to 0.018/min.[1] The transfer coefficient, k_{va}, will therefore have a value between 0.19 and 0.31/day. The lower of these values is still slightly above the value, 0.163/day, found in this paper for the total transfer coefficient out of the vitreous body, k_{vo}. Two conclusions may be drawn: (1) that virtually all the activity in the vitreous body leaves it by way of the anterior chamber; (2) that the value for the outflow coefficient of the aqueous humor is of the order of 0.010/min.

This equation can also be used to calculate the maximum value of k_f in individual eyes in the form:

$$k_f < k_{vo} \frac{V_vC_v}{V_aC_a}$$

In Table 1 are chosen those eyes which had the best defined values of k_{vo} and the least reaction to the injection or the longest recovery from the reaction. It is seen the average value of k_f is 0.0085 min.

It will be noted that the chemical state of the radioactivity is not important for these arguments but only its total amount in each fluid. Again, if any activity left the vitreous body other than through the anterior chamber, or left the anterior chamber other than by flow, the discrepancy between k_{va} and k_{vo} would be greater.

Movement in the vitreous body

The movement of Na^{24} from the vitreous body into the anterior chamber was at a rate

TABLE 1

CALCULATION OF OUTFLOW COEFFICIENT OF AQUEOUS HUMOR FOR INDIVIDUAL EYES

Rabbit	Time of Experiment (days)	k_{vo}(/day)	C_a/C_v	k_f(/min.)	Remarks
3	15	0.131*	0.078	0.0069	No reaction noted
8	11	0.196	0.048	0.016	Slight vitreous opacities. No other reaction
17	21	0.149	0.072	0.0085	Small traumatic cataract. No reaction noted
28	19	0.161	0.15	0.0044	Slight vitreous opacities. No other reaction
30	19	0.173	0.082	0.0086	Slight opacities. No other reaction
40	15	0.170*	0.083	0.0085	Strong flare gone 6th day. Tonometry, tonography normal 6th day
42	13	0.143*	0.084	0.0070	Slight flare 1st day only. Tonometry, tonography normal 2nd day

Mean = 0.0085.

* The activity of the control eye was subtracted. In the other animals a seven percent correction has been made to k_{vo}.

consistent with it being limited only by the slowness of its diffusion in the fluid of the vitreous body itself.[8] The structural framework of the vitreous body or hyaloid membrane did not seem to restrict the diffusion of the ion. It is interesting to calculate whether the same conditions hold for the movement of albumin. A more exact calculation is feasible here, since there is not the complication of a direct loss from the vitreous body to the blood across the retina, such as occurs with Na^{24}.

The computation by strictly mathematical procedures of the diffusion across the surface of an irregular body such as the vitreous would be very laborious. It was decided therefore to derive the outflow coefficient directly by means of a thermal analogue. Accordingly, a model of a sector of the vitreous body of the rabbit was cast in lead and shaped with a file to be in scale with the dimensions shown in the literature[4,5] (fig. 3). The surface corresponding to the vitreous-posterior chamber interface was passed through a hole cut in a sheet of rubber and was sealed to it with adhesive (fig. 4). The remaining surfaces were insulated with blocks of "styrofoam" shaped to fit them.

A small thermistor connected to a recording voltmeter was held in contact with the model at a point, A, corresponding to the center of the vitreous body. Hot water was run over the exposed surface until the model reached a suitable temperature. Cold water at a fixed temperature was then run over the surface and the change in temperature of the model recorded with the thermistor until a steady level was reached. The difference between the temperature of the model and this level plotted against time on semilogarithmic paper gave a linear relationship. The laws governing the diffusion of heat and the diffu-

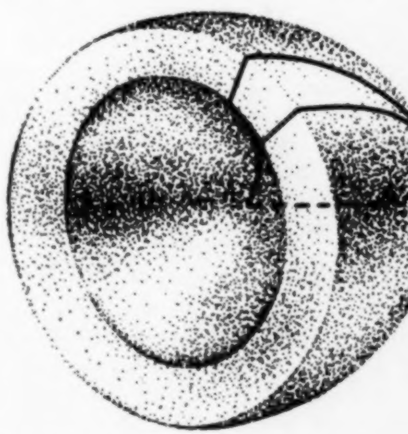

Fig. 3 (Maurice), Diagram showing segment of vitreous body used for thermal analogue.

PROTEIN DYNAMICS IN THE EYE 367

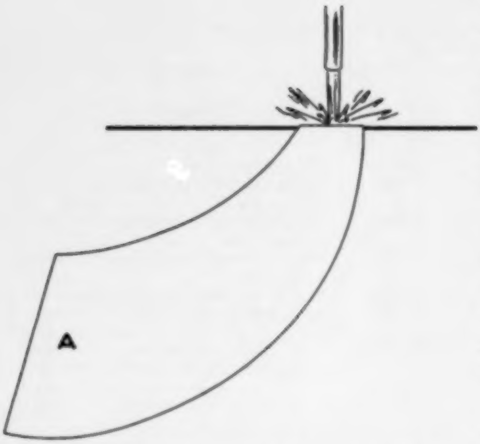

Fig. 4 (Maurice). Diagram illustrating experimental procedure followed in analogue experiment.

sion of dissolved substances being formally identical, this model accurately simulates the process of free diffusion out of the vitreous body into the posterior chamber.

Only a sector of the vitreous need be represented since, under these conditions, there is no flux of heat or labelled proteins across the boundary of one sector and another. If the decay constant of the temperature drop is k_m, the transfer coefficient of albumin out of the eye would be given by:

$$k_{vo} = k_m \frac{D_v}{\kappa_m}\left(\frac{d_m}{d_v}\right)^2$$

Here, d_v and d_m represent the equatorial diameters of the vitreous body,[4] 1.7 cm., and the model, 26 cm. D_v is the diffusion constant of albumin in free solution at 37°C. For horse serum albumin it has been found to be 6.1×10^{-7} cm.^{2}/sec. at 20°C.,[6] corresponding to a value of 8.4×10^{-7} cm.^{2}/sec. at the higher temperature; there is unlikely to be a large species difference in the size of the molecule. κ_m is the thermal diffusivity of the lead. This was measured before the model was cast, when the metal was in the form of a rectangular block. Exactly the same procedure was used as for the model, and in this case it was possible to calculate how the temperature at the end face of the block should change with time.[8] The temperature recorded by the thermistor followed the theoretical relationship closely when a value of κ_m of 0.232 cm.^{2}/sec. was adopted. This is very near the value of κ_m for lead quoted in the literature,[9] 0.236 cm.^{2}/sec.

Experimentally, a value 0.120/min. was found for k_m. It may be noted in passing that the size of the vitreous-posterior chamber interface does not critically affect this figure; it was purposely made 20-percent oversize in the casting and experiments made before it was filed down to size led to a value of k_m of 0.126/min. Inserting the values in the equation leads to a figure of 0.148/day for k_{vo}. This is sufficiently close to the experimentally found value, 0.163/day, to justify the hypothesis that the albumin leaves the vitreous body by free diffusion. Uncertainties which could account for the residual differences are to be found in the diffusion constant of the albumin and in the size of the eyes, which were not measured but were in many cases from immature animals. On the other hand, it was concluded[5] from measurements on the conductivity of the vitreous body that the diffusivity of all ions would be reduced by 10 percent from the free solution values. If this were true it would increase the disparity found above.

Another hypothesis which can be readily tested with the model is whether the rate at which the albumin first arrives in the aqueous humor is consistent with its free diffusion in the vitreous body. This is simulated by briefly heating the model at the point A and measuring the change of temperature at the anterior surface with the thermistor. It is assumed that the increase in temperature will be proportional to the rate at which the albumin enters the anterior chamber when the time scale is multiplied by the factor

$$\frac{D_v}{\kappa_m}\left(\frac{d_m}{d_v}\right)^2.$$

The results indicate that the rise in concentration in the aqueous humor would be as

shown by the curve drawn in Figure 1. It is seen that it does not agree with the few experimental points taken in this early period. The explanation may well be that the albumin solution was not injected exactly on the axis of the eye as the analogue requires. If the injected volume was off the axis it would initially have a short diffusion path to the anterior chamber and therefore would appear sooner.

Comment

These results suggest that there is no fluid flow within the vitreous body and that its structure offers no important resistance to the diffusion of albumin within it. This is not surprising when the looseness of this structure is considered.[5] It contains about r part in 10,000 of a structural protein which appears, under the electron microscope, to be divided into fibrils 20 mμ in diameter. The average distance between the fibrils should therefore be of the order of 2.0μ, several hundred times greater than the diameter of the albumin molecule. In addition, there is present about three times this concentration of a mucopolysaccharide; though this may slightly increase the viscosity of the fluid between the fibrils it is unlikely otherwise to resist the diffusion of protein. Little is known definitely about the hyaloid membrane. It is certainly more condensed than the rest of the vitreous body but there is no evidence to suggest that its fibers are sufficiently close to form a membrane of reduced permeability.

The value of the aqueous humor outflow derived from the experiments is considerably lower than that found by most other methods. However, in these, errors are compounded by elaborate mathematical treatments and uncertain assumptions.[1] The assumptions required in the method of this paper are few and if they were incorrect would result in an even lower value for the outflow.

Summary

Iodine[131] labelled albumin was injected into the vitreous body of rabbits and its rate of loss from the eye determined with an external counter. At various times after the injection the concentration of active material in the vitreous and aqueous humors was compared.

The results show that virtually all the protein leaves the vitreous body by way of the anterior chamber. They show, also, that the outflow coefficient of the aqueous humor of these animals is about 0.0085/min.

A thermal analogue to the diffusion conditions in the vitreous body was constructed. The comparison suggests that the vitreous humor is stagnant and that there is virtually unrestricted diffusion of the albumin within the vitreous body and across its boundary with the aqueous humor.

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Medical Center (22).

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Discussion

Dr. Harry Green (Philadelphia): It has become apparent that the diffusional exchange of some test substances between the aqueous humor of the posterior chamber and the vitreous humor may significantly affect the analysis of the dynamics of exchange across the blood-aqueous barriers. By previously employing the technique of injecting tracer amounts of Na^{24} into the vitreous humor of the rabbit eye, Dr. Maurice has corroborated the findings of Davson, Kinsey and Palm, and Friedenwald and Becker, and, in addition, has shed light upon the dynamics of exchange of the radioactive sodium between the aqueous humor and the vitreous humor and the vitreous humor and the blood plasma.

In the present investigation Dr. Maurice has initiated the study of the dynamics of a particularly difficult substance to handle, but a very essential test substance, namely, protein. His results suggest that diffusional exchange with the vitreous humor be considered in evaluating the exchange of protein between the aqueous humor and the plasma.

I am sure Dr. Maurice is planning to continue these studies in an attempt to determine, among other things, the mechanism whereby proteins enter the aqueous humor, and indeed whether they enter the posterior chamber only from the plasma, as Seidel suggested many years ago.

In this connection I should like to ask Dr. Maurice whether analysis of the posterior chamber aqueous humor also would be of more direct interest than anterior chamber analysis alone.

I wish to commend Dr. Maurice for his opening up of this very important field of aqueous humor studies, and I am looking forward with great interest to the results of his continued researches. I am also particularly impressed with his ingenious adaptation of physical models, in this case a thermal analogue for the simplification of a very complex organ.

Dr. V. Everett Kinsey (Detroit): If I understood Dr. Maurice correctly, he arrived at the conclusion that the outflow of the aqueous in the anterior chamber is about half of that cited by various workers using other methods.

Is the alternative interpretation likely, namely, that the outflow for protein from the anterior chamber is less than it is for other substances?

Dr. David M. Maurice (closing): I would like to thank Dr. Green. He realizes that I chose the easy experiments to do, not the difficult ones.

I would like to measure how fast protein enters the posterior chamber but it is extremely difficult to do so; there is so little protein in the fluid and the volume of the posterior chamber is so small. I have met with the greatest difficulty in obtaining sufficient material even in the anterior chamber, without making the rabbits so radioactive that they were a menace. I hope someone can suggest another way of doing it.

Dr. Kinsey's question was whether the outflow of proteins is less than other substances. I find this difficult to believe. First of all, there is the size of the outflow channels, which has now been established by perfusion methods and is many hundreds of times larger than the diameter of the protein molecule. Then there is the question of how the substance would get back into the anterior chamber from the site of filtration—well, I suppose this is a possibility that could be examined by cutting off the anterior part of the eye and seeing if there is any excess of radioactivity. I have never done that.

CHANGES IN PROTEINS AND PROTEIN SYNTHESIS*

In tryptophane deficiency and radiation cataracts of rats

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New York

It has been previously shown that when five- to six-week-old rats are put on a standard galactose diet with 35 percent galactose, the net synthesis of soluble proteins during the first six days of galactose feeding is decreased by 60 to 100 percent.[1] The deposition of albuminoid, on the other hand, proceeds during this time interval either at a normal rate or it is somewhat slowed down. This decrease in the albuminoid formation can be accounted for under the assumption that the synthesis of this protein takes place from soluble proteins and its rate is dependent upon the concentration of the latter.

In addition, changes in the chemical nature of soluble proteins also take place which

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manifest themselves in the appearance of a characteristic protein which on oxidation with $K_3Fe(CN)_6$ becomes insoluble. A certain amount of such a protein was found always to be present in lenses of rats not older than three months, its amount decreasing with increasing age. This suggested that this new protein is an intermediate stage in the differentiation of lens proteins and may be identical or similar to the so-called ambryonal protein found by François[2] and his associates in the lenses of embryos and the nucleus of adult animals.

As the inhibition of the synthesis of soluble proteins was not accompanied by a corresponding decrease in the total volume of the lens, the concentration of soluble proteins in the lenses of galactose fed rats decreased significantly with progressing feeding.

All these observations suggested that the formation of galactose cataracts may be the result of two processes, namely, the disruption of the homogeneity of a gel or gellike formation consisting of soluble proteins due to the decrease in the concentrations of these proteins, and at the same time disturbances of the lenticular metabolism due to the increase in albuminoid. The latter, according to Mörner's[3] findings, can be assumed to be localized on the membranes of the lenticular fibers and its quantity thus influences the permeability of these membranes for nutrients. The resulting disturbance of the lenticular metabolism might affect the co-ordination of growth and differentiation processes of the rapidly growing lens of young rats and thus finally lead to a disruption of structural continuity and optical homogeneity.

In most experimental cataracts so far produced in laboratory animals, the age of the animals is of considerable significance in the sense that the younger the animal, the greater its susceptibility to various forms of experimental cataracts. It seemed, therefore, possible that the same changes in the chemistry and synthesis of lens proteins, which have been observed during the formation of galactose cataract, may also have a pathogenic significance in other forms of experimental cataracts. A series of experiments, therefore, were carried out in which the synthesis of lens proteins was determined on rats in which cataracts were produced either by tryptophane deficiency or X-ray irradiation. The present report deals with some of these experiments.

I. Experiments on tryptophane-deficiency cataract

1. Animal material and experimental procedure

The experiments were carried out on Rockland Farm rats of 34 to 37 days of age. Each batch of experimental animals consisted of seven to 11 animals which received a tryptophane-free diet, according to Buschke,[4] with acid hydrolyzed casein as protein source. The animals of control and experimental groups were selected in such a way that the initial weights of whole groups did not differ significantly. The control animals received the same diet with a supplement of 2.25 gm. of 1-tryptophane per 1,000 gm. of diet. The food consumption of the experimental animals was significantly lower than that of control animals (4.0 gm. average against 8.49 per day), and as a result these animals, instead of gaining weight, lost some of their initial weight. After about four weeks, however, the body weight stabilized at a level about 25 to 30 percent below the initial value, and did not increase during a following period of 11 days.

The reduced food intake in experimental animals made it necessary to carry out control experiments in which one group of animals on a normal pellet diet was allowed to eat ad libitum, while another group of animals was kept on an identical but calorically restricted diet.

The lenses of animals were examined with the slitlamp at various time intervals to determine the time at which initial cataractous changes could be observed. Such changes mostly appeared in form of subcapsular opacity or haze, which first could be ob-

served only in a certain percentage of the experimental animals. The changes, appeared for the first time in one group of animals after 19 days of tryptophane-deficient diet. The cataractous changes were often accompanied by vascularization of the cornea. These observations agreed with the data of Buschke obtained in rats on tryptophane-deficient diet.

At the end of every experimental period the animals were killed by ether, the lenses removed, wiped dry with filter paper, and weighed in a weighing bottle. A 10-fold amount of the weight of the lenses of distilled water was then added to each batch of the lenses in two steps:

First a homogenate of lenses was made in one cc. of water by crushing them completely with a glass rod, whereupon the remaining volume of water was added to the homogenate and the latter transferred into plastic centrifuge tubes cooled to 0°C. in ice water. The homogenate was then centrifuged at 13,000 rpm for one hour in a Spinco preparative ultra centrifuge and the supernatant containing the soluble proteins poured off. The weighing bottle which contained a small amount of homogenate was then rinsed over with distilled water into the centrifuge tube, which now contained all of the water insoluble material of the lens. This material was washed three times with distilled water, once with saline, and then twice with five-percent TCA. It was then rinsed over from the centrifuge tube into a graduated 10-cc. cylinder with a ground glass stopper, dissolved by adding 2.5 cc. of 2 N sodium NaOH, and the solution filled up to 10 cc. The same cylinder was used for dissolving the albuminoids from the experimental lenses and controls. The solutions were frozen over night and used the next day for analysis. For this purpose, one cc. of the solution of soluble lens proteins was diluted with three cc. of water and made up to five percent of TCA with 100-percent TCA solution. The precipitate was left for a few hours in the refrigerator, centrifuged off, washed with five-percent TCA, and dissolved in 0.5 N NaOH to a volume of 10 cc. This solution as well as that of the albuminoid was used for the determination by micro-Kjeldahl of the soluble and insoluble proteins of the lens.

To test the accuracy of this procedure soluble proteins were determined in seven duplicate sets of 10 lenses, each set formed either from right and left lenses of the same animal, respectively, or from two corresponding groups of animals of the same weight. In four sets the values differed by less than one percent, the other differences being 1.3, 2.2, and 3.8, respectively.

The remaining supernatant of the lens homogenate was used for the determination of the effect of the oxidation by $K_3Fe(CN)_6$ of the soluble lens proteins on their solubility. To this end, 0.2 to 1.0 cc. of the solution was mixed with 1.0 cc. of a phosphate buffer of pH 7.4, 1.5 cc. of ferricyanide of M/400, and filled up with water to a volume of 3.5 cc. Duplicate samples were taken from the experimental as well as control solutions, and one sample was taken for the determination of the absorption due to the protein itself. In this sample the solution of ferricyanide was replaced by distilled water.

All samples were incubated simultaneously in a water bath of 37°C. for time intervals of 1.5 to three hours, and their transparency was then measured at 480 mμ in the Beckman spectrophotometer. It has been established in previous experiments that the ratios of transparencies measured in this way corresponded within 10 percent to the amounts of protein which were precipitated and were determined by micro-Kjeldahl.

2. Synthesis of soluble proteins and of albuminoid

Seven experiments were carried out with cataracts induced by a tryptophane-deficient diet in which the experimental periods varied between 10 and 49 days. The results of this series of experiments are listed in Tables 1 and 2.

Slight cataractic changes in the lenses of

TABLE 1

PROTEIN SYNTHESIS IN LENSES OF RATS ON TRYPTOPHAN-FREE DIET WITH AND WITHOUT SUPPLEMENT OF L-TRYPTOPHAN

Exp. No.	Days Since Start of Experiment	No. of Lenses	Age at Time 0	Diet (tryptophan)	Body Weight	Wet Weight in mg./lens	Soluble Proteins in mg./lens	Insoluble Proteins in mg./lens	Soluble Proteins mg. in 100 cc. of lens	Soluble / Insoluble Proteins
I	0	20	35		107.5	23.5	7.30	1.16	44.1	6.2
	12	16		With	131.0	25.6	8.60	1.88	37.0	4.57
	12	16		Without	96.8	25.8	8.25	1.88	35.1	4.38
Group A	19	16		With	124.5	29.0	9.3	2.16	35.2	4.34
	19	19		Without	89.2	24.7	7.8	2.30	34.4	3.38
Group B	19	16		With	128.0	28.8	9.3	2.18	35.5	4.23
	19	20		Without	87.5	23.7	7.55	2.28	35.1	3.31
II	0	22	36		94.0	23.3	6.42		30.0	4.36
Group A	10	20		With	121.3	26.4	8.10			
	10	20		Without	88.0	24.0	7.6			
Group B	10	20		With	122.4	25.8	8.18	1.84	36.3	4.44
	10	20		Without	85.0	24.3	7.71	1.79	34.0	4.30
III	0	20	35		87.2	22.5	6.63	1.31	31.7	5.0
	12			With		26.4	7.43	1.95	30.6	3.85
	12			Without		25.7	6.87	1.99	30.3	3.45
IV	0		35		67.0					
	19	14		With	88.0	27.8	7.6	1.90	30.9	4.0
	19	16		Without	54.6	24.8	6.3	1.93	30.7	3.26
	38	14		With	118.6	32.4	9.6	2.96	35.5	3.24
	38	14		Without	52.4	24.9	6.85	2.50	32.5	2.74
V	0	20	37		94.2	24.6	7.25	1.60	34.0	4.53
	41	22		With	64.0	36.2	10.8	3.75	36.1	2.88
	41	14		Without	173.0	28.0	8.0	3.0	34.6	2.67
VI	0	0	35		97.0	23.3	7.60		37.8	
	49	30		With	200.0	35.7	11.0	4.0	37.8	2.75
	49	34		Without	65.6	30.6	8.0	3.2	31.4	2.50
VII	0	40	37		67.5	20.0	6.1	1.73		
	28	18		With	127.0	29.4	9.0			
	28	30		Without	51.0	24.4	7.0			
	39	14		With	146.0	31.6	9.8	2.87	37.2	3.4
	39	26		Without	50.0	23.8	6.56	2.58	33.1	2.5

animals on tryptophane-deficient diet could first be noticed after 19 days at which time about one third of the animals showed posterior subcapsular central opacities. After 28 days, all animals showed such opacities, and in some of them opacities appeared in the anterior part of the lens. Any changes in protein synthesis in time intervals up to 19 days can, therefore, be regarded as precataractous and up to 28 days as associated with initial cataractic changes.

As can be seen, in three experiments (I-III), a decrease in soluble proteins appeared already after 10 to 12 days. The decrease

TABLE 2

EFFECT OF $K_3Fe(CN)_6$ OXIDATION ON THE SOLUBILITY OF SOLUBLE LENS PROTEINS OF RATS ON TRYPTOPHAN-FREE DIET WITH AND WITHOUT SUPPLEMENT OF L-TRYPTOPHAN

Exp. No.	Days since Start of Experiment	Age at Time 0	Diet (tryptophan)	Body Weight	Time of Incubation (hr.)	% Protein in Homogenate	$D_{450}\times1000$ of Solution after Incubation
I	12	35	With	131.0	2½	3.20	385
			Without	96.8		2.93	282
IV	19	35	With	88			
	19		Without	54.6			
	19		With		2½	2.12	815
	19		Without		2½	1.93	572
	38			118.6		2.85	1308
	38			52.4		2.61	1058
VI	49	35	With	65.6	3	2.97	476
	49		Without	20.0	3	2.48	415
VII	28	37	With	127.0	2½	2.79	686
	28		Without	51.0		2.62	402
	39		With	146.0		2.90	718
	39		Without	50.0		2.50	597
VIII	12	35	With	112.3	2½	2.23	826
	12		Without	75.0		2.30	660
IX	12	35	With	108.420	2½	2.63	794
	12		Without	75.016		2.47	662

varied between 4.0 and 7.5 percent. After 19 days of tryptophane feeding in three groups of animals (Experiments I and IV), the decrease was 16 to 19 percent which was far above the possible error. This decrease represented an inhibition of at least 75 percent of the rate of synthesis. With progressing feeding, the degree of inhibition remained at about the same level as that observed after 19 days.

The synthesis of albuminoid present a completely different picture. There was no significant change whatsoever in lenses of tryptophane-deficient animals after 19 days or earlier. After more than five weeks, there was a decrease of 10 to 20 percent. It must be noted, however, that this decrease in albuminoid coincides with an on the average 20 to 25 percent lower content in soluble proteins in lenses of tryptophane-deficient animals in the period after 19 days.

Finally, marked changes also appear in the wet weight of lenses of experimental animals. These changes, however, became significant only after a period of 19 days when they reached between 11 and 18 percent of the corresponding control values. In later stages of the tryptophane deficiency, the differences in the wet weight between experimental and control lenses were somewhat higher than after 19 days, varying between 18 and 25 percent. This increase in the difference in the wet weight after 19 days appears to be due to the fact that the wet weight in the experimental animals changes very little if at all after the 12th day of feeding while the wet weight of the controls increases continuously during the whole experimental period.

The changes in the synthesis of soluble proteins and in the wet weight of experimental lenses suggested changes in the concentration of soluble proteins within the lens fibers which might be of significance as a pathogenic factor in tryptophane-deficiency cataract. The concentration of soluble proteins in the water phase of the lens was, therefore, estimated in the following way:

The approximate water content of the lens was determined by subtracting the weight of soluble proteins and albuminoid from the wet weight of the lens and subtracting from this difference one percent of the wet weight for the approximate weight of electrolytes. The volume of the lens was then estimated by adding the volume of proteins obtained by dividing their weight by the average specific density 1.25. The concentration of the soluble proteins in this calculated volume of the water phase was then calculated and is listed in Column 9 of Table 1.

As can be seen, this value is significantly lower starting with the 28th day of tryptophane feeding. After 19 days, it appears lower, but by an insignificant amount. On the other hand, the decrease in soluble proteins under the influence of tryptophane deficiency and the significantly different behavior of the albuminoid leads to a large shift in the ratio of these two proteins, as can be seen from the last column of Table 1. This marked shift appears already after 12 days and becomes even more pronounced with progressing tryptophane deficiency.

3. The effect of oxidation by $K_3Fe(CN)_6$ on soluble lens proteins of tryptophane-deficient animals

In four of the seven experiments listed in Table 1, we also determined the amount of the characteristic protein which precipitates after oxidation with $K_3Fe(CN)_6$ and which was previously shown to be a constituent of the beta crystalline protein.[1] In addition, such determinations were carried out on homogenate supernatants of lenses of two other groups of tryptophane-deficient rats on which no further analyses of proteins could be carried out.

The results of these experiments are reported in Table 2.

As can be seen from Column 8 of this table, in all six experiments in the time interval of up to 28 days the experimental lenses showed a marked decrease in the amount of protein which precipitated after 1.5 to 3.0 hours, of incubation. Although the concentration of protein was somewhat smaller in experimental homogenates than in controls, in almost every case these differences are far too small to explain the differences in the amounts of precipitated proteins between experimental samples and controls.

In addition, the amount of $K_3Fe(CN)_6$ reduced in experimental samples (measured in the supernatant after precipitation of the protein by TCA) was not significantly smaller than that in controls. This indicates a slight increase in the availability of oxidizable groups of lens proteins under the influence of tryptophane deficiency. That means that a significantly smaller fraction of the oxidated proteins was precipitated. With the progressing tryptophane deficiency the difference between experimental and control samples not only did not increase, as has been found in galactose cataract, but on the contrary tended to disappear or to become insignificant. Tryptophane cataract in this respect differs significantly from galactose cataract.

4. Effects on beta crystalline synthesis

The protein which after oxidation with $K_3Fe(CN)_6$ becomes insoluble was shown to form part of the beta crystalline fraction of the lens.[1] The decrease in this fraction in tryptophane deficiency suggested that it may be due to a more pronounced inhibitory effect on the synthesis of beta crystalline than on that of other lens proteins. The proteins of homogenates of lenses of tryptophane-deficient animals and corresponding controls were, therefore, fractionated in Experiments IV and V. This fractionation was carried out by adjusting the pH of the homogenate with acetic acid to pH 5.2, centrifuging off the precipitate and dialyzing a measured aliquot of the supernatant against repeatedly changed large volumes of distilled water of a pH of about 5.0. Most of the remaining alpha crystalline came out of solution under these conditions.

The total fluid inside the bag was then

filled up to a certain volume, the supernatant centrifuged off, and its protein content, consisting mainly of beta crystalline with only small amounts of other proteins, was determined in both dialysates. While in the two controls of Experiments IV and V, 66.5 and 69.5 percent of the total protein was found in the dialysate, the corresponding percentage values in lenses of tryptophane-deficient animals were 62 and 64.5. The synthesis of beta crystalline, therefore, appears to be stronger inhibited than that of alpha crystalline. But the decrease in the first protein is not sufficient to account for the decrease in the synthesis of total soluble protein.

5. Effect of reduced food intake on the synthesis of lens proteins

Two control experiments were carried out to test the possibility that the effects of the tryptophane-free diet may be due to reduced food intake rather than the deficiency itself. In these experiments, one batch of eight to 10 rats received the standard pellet diet ad libitum while another batch received an amount of pellets chosen in such a way that the animals either did not gain weight at all or even showed a slight loss of weight in the beginning of the experiment.

Animals on restricted diet and controls were killed after 10 and 20 days and soluble proteins and albuminoid were determined. The results of these experiments are presented in Table 3. As can be seen, the soluble proteins in animals on restricted diet either did not decrease at all, or the decreases was within the limits of error of the determinations.

As the insoluble proteins decreased in all cases by about five to 10 percent, the ratio soluble to insoluble proteins slightly increased in all three cases. Although the differences are small, they may be significant and it should be noted that in every one of the 10 groups of animals on tryptophane-deficient diet so far tested this ratio decreased without exception starting with the 12th day of feeding.

II. Experiments on X-ray cataracts

Six experiments of this kind were carried out. In two experiments the right eye of the animals received 2,000 r in 2.5 minutes (KV 107-108, 30 M amp., 19.5 cm. distance, 0.5

TABLE 3

Effect of restricted pellet diet on synthesis of lens proteins of rats

Exp. No.	Days since Start of Experiment	No. of Lenses	Age at Time 0	Diet	Body Weight	Wet Weight in mg./lens	Soluble Proteins in mg./lens	Insoluble Proteins in mg./lens	Soluble / Insoluble Proteins
I	0	20	35		88.0	20.0	6.66	1.27	5.28
	10	16		Normal*	130.0	25.0	8.58	1.70	5.05
	10	16		Restricted†	77.0	25.0	8.25	1.54	5.36
	20	12		Normal*	166.0	28.1	9.27	2.15	4.31
	20	12		Restricted†	99.0	28.0	9.15	2.10	4.36
II	0	20	35		108.0	22.0	7.40	1.28	5.78
Group A	20	20		Normal*	190.5	26.0	9.04	2.19	4.13
	20			Restrict.‡	106.0	26.1	8.78	2.01	4.38
Group B	15	20		Normal*	190.5	27.7	9.34	2.09	4.47
	15	20		Restrict.‡	106.0	27.5	9.49	2.11	4.50

* 10g per day.
† 7g per day.
‡ 8g per day.

cm. openings), in four others, 3,000 r in 4.13 minutes. The left eye was shielded. The animals were anesthetized before irradiation with 5.5 mg. nembutal per 75 gm. body weight. In four of the experiments the animals were divided into two groups which were killed at two different time intervals. In special control experiments a separate batch of animals which were not irradiated at all were killed at the same time as the irradiated animals.

These control experiments showed that the protected left eye was not affected by the irradiation, nor was there any significant difference between the left and right eyes of nonirradiated animals. In animals which received only 2,000 r, in seven out of eight irradiated lenses, a suggestion of haze at earliest stage could be observed with the slitlamp 25 days after irradiation (Experiment II). One lens at that time was completely clear. In animals which received 3,000 r accentuation of the suture and subcapsular opacities of varying density corresponding to early stages of cataract were observed after 22 days (Experiment V). After 16 days in the same experiment only one third of the lenses showed early cataractous changes. Determinations of the lens proteins and of the turbidities after $K_3Fe(CN)_6$ oxidation were carried out in the same way as in the case of tryptophane deficiency.

1. Changes in synthesis of soluble proteins and albuminoid in irradiated lenses

Changes in soluble protein synthesis in irradiated lenses were determined at time intervals ranging from 14 to 84 days after irradiation. The results are presented in Table 4. In animals irradiated with 3,000 r a decrease of six percent after 14 days and of 9.3 percent after 26 days took place.

In animals irradiated with 2,000 r, there was a decrease of about nine percent in the soluble proteins after 28 days, at which time there were observed only very early cataractic changes. The decrease went up to about 14 percent after 38 to 60 days (Experiments I and II). At this lower dose the decrease in the rate of synthesis appeared fairly constant in successive stages of the cataractic process, and amounted to 30 to 50 percent of the values in control eyes.

In animals irradiated with 3,000 r, the inhibition in early stages was higher and finally led with progressing cataract to a loss of soluble proteins. The albuminoid did not show any significant changes except in one single batch of animals (Experiment IV) where 43 days after irradiation the decrease in albuminoid was about six percent. The concentration of soluble proteins calculated in the same way as described above for lenses from tryptophane-deficient rats showed a significant decrease only after 28 days and 26 days in animals which received 2,000 and 3,000 r, respectively. The ratio of soluble to insoluble proteins decreased also significantly after 29 and 24 days, respectively.

2. Effect of oxidation by $K_3Fe(CN)_6$ on lens proteins after irradiation

The formation of beta crystalline which precipitates after oxidation was slightly increased, as can be seen from Table 5, after 24 days in animals which received 3,000 r. After a dosage of 2,000 r, there was no significant change in the amount of precipitable protein even 60 days after irradiation. Percentually, the increase in precipitable protein did not become more pronounced with progressive cataract, and in Experiment I, 26 days after irradiation with 3,000 r, the increase was less than seven percent.

Discussion

The results reported in this paper indicate that in all three forms of experimental cataract, so far investigated, namely, galactose, tryptophane deficiency, and radiation cataract, the synthesis of soluble and insoluble lens proteins is affected in an identical way insofar as in all three forms of cataract the net synthesis of soluble proteins is decreased while that of albuminoid is either not changed

TABLE 4

PROTEIN SYNTHESIS IN LENSES OF IRRADIATED RATS

Right eye irradiated; left eye shielded

Exp. No.	Body Weight	Days Since Start of Experiment	No. of Lenses	Age at Time 0	Irradiation	Wet Weight in mg./lens	Soluble Proteins in mg./lens	Insoluble Proteins in mg./lens	Soluble Proteins mg./100 cc. lens	Soluble / Insoluble Proteins
I		0		35	2000 r					
	200.7	29	10		nonirradiated	33.0	9.97	3.04	32.9	3.28
			10		irradiated	32.5	9.57	3.25	32.3	2.95
		60	11		nonirradiated	40.8	12.8	4.86	34.4	2.63
	304.0		11		irradiated	38.3	11.0	4.60	31.2	2.39
II	79.0	0	21	35	2000 r	22.2	7.22			
			21			22.2	7.20			
	170.0	28	13		nonirradiated	31.6	9.13	3.01	32.2	3.0
		28	13		irradiated	30.1	8.36	2.98	30.9	2.8
	208.0	84			nonirradiated	41.8	11.9	4.9	32.0	2.4
		84			irradiated	37.3	10.3	4.9	31.1	2.1
III	106.5	0	20	35	3000 r	23.5	7.76	121	34.2	6.4
	149.0	14	8		nonirradiated	26.9	8.52	2.50	39.8	3.4
			8		irradiated	26.4	8.40	2.46	34.7	3.4
		26	9		nonirradiated	30.7	10.10	2.69	36.2	3.74
			4		irradiated	31.5	9.17	2.52	31.4	3.64
IV	81.0	0	12	34		22.4	7.29			
			12			22.4	7.02			
	197.0	36	14		3000 r	34.0	10.30	2.95		
					nonirradiated				33.8	3.4
		36	14		irradiated	29.0	8.43	2.95	32.4	2.8
	231.0	43	18		nonirradiated	35.8	11.1	3.77	34.9	2.9
		43	9		irradiated	33.2	8.19	3.54		
		43	9*		irradiated	29.0	3.2		27.6	2.3
V	67.0	0	20	35	3000 r	20.2	6.06	1.76		
			20			19.8	6.14	1.79		
	202.0	24	14		nonirradiated	30.1	9.04	2.25	33.4	4.0
		24	14		irradiated	28.4	8.50	2.44	33.3	3.5
	242.0	37	20		nonirradiated	33.9	9.7	2.96	32.0	3.3
		37	15		irradiated	29.4	8.0	2.84	30.3	2.8
		37	5*		irradiated	37.7	6.6	2.98	18.9	2.2

* Mature cataract.

at all or decreased to a much smaller degree.

The result of these shifts in the rate of synthesis in lens proteins is a decrease in the ratio of soluble proteins to insoluble proteins which becomes more pronounced with progressing cataract. Although in all three cataractous processes there also is a marked slowing down in the increase of wet weight and volume of the lens, the latter does not keep pace with the decrease in synthesis of soluble proteins. As a result there is a decrease in the concentration of soluble lens proteins.

These similarities appear noteworthy as it appears most probable that the three cataractogenic agents have different points of attack as far as chemical and morphologic substrates in the lens are concerned. These similarities in the effects of the three cataractogenic agents on the net protein synthesis suggest, therefore, that these effects play a role as pathogenic factors in all three cataractous processes.

In an attempt to analyze the role of such changes of protein synthesis in mechanisms underlying the formation of cataract, it is first necessary to pay due attention to the fact that the ocular lens is structurally a morphologically in homogeneous organ consisting of successive layers of cells of very different age and degree of differentiation as well as of continuously changing chemical

TABLE 5

EFFECT OF OXIDATION BY K_3FeCN_6 ON SOLUBLE LENS PROTEINS OF IRRADIATED RATS

Right eye irradiated; left eye shielded
One cc. of lens homogenate + one cc. of 1.0 M phosphate buffer 41.5 cc. M/1400 K_3FeCN_6

Exp. No.	No. of Lenses	Days Since Start of Experiment	Age at Time 0 In Days	Irradiation	Time of Incubation	Percent Protein in Homogenate	$D_{400} \times 1000$ of Solution after Incubation
I		29		2000 r			
				irradiated		1.96	711
				nonirradiated		2.04	701
II			35	3000 r			
	13	28		irradiated	3 hours	1.94	989
	13	28		nonirradiated		2.12	915
	10	84		irradiated		2.62	270
	10	84		nonirradiated		2.76	230
				3000 r			
III		26		irradiated		1.39	478
				nonirradiated		1.60	508
IV			34	3000 r			
	14	35		irradiated	2½ hrs.	2.23	1116
	14	35		nonirradiated		2.77	945
	9	43		irradiated		2.11	882
	18	43		nonirradiated		2.39	620
V			35	3000 r			
	14	26		irradiated	2½ hrs.	3.01	930
	14	26		nonirradiated		2.84	750
	15	37		irradiated		2.65	1028
	20	37		nonirradiated		2.48	873

composition along a gradient from the surface toward the center of the lens. The synthesis of soluble lens proteins takes place to a large extent in the equatorial part and on the surface of the lens and is due to a differentiation of the equatorial lens epithelium into lens fibers.

It seems reasonable to assume that these newly formed surface fibers contain only very small amounts of albuminoid or none at all. This can be deduced by analogy with the conditions in the lens of cattle where the content in albuminoid in lenses of very young animals is much lower than in older animals and, furthermore, much lower in the superficial cortical lens fibers than in those more centrally located.[5]

The same conclusion can be drawn from the consideration of the kinetic of the albuminoid formation in lenses of young rats. The rate of formation of this insoluble protein decreases continuously with age after about five weeks of the postnatal life.[6] If the rate in the first four or five weeks of the postnatal life would follow the same pattern the amount of albuminoid in lenses of five-week-old rats would have to be several times as high as it really is. In reality, however, this amount is about equal to that produced between the fifth and eighth week of postnatal life. In the beginning, therefore, of the postnatal period the formation of albuminoid follows an autocatalytic course.

Thus, it appears reasonable to assume that freshly formed lens fibers contain only minimum amounts of albuminoid and the latter increases with the progressing age of the fiber in the rat as had been found to be the case in cattle lenses.

Although one part of the soluble lens proteins in the growing lens is produced in the fibers newly formed from the epithelium, there can be little doubt that another part is formed in deeper lens fibers, at least those which contain still nuclei. This can be inferred from the ratio between the increase

in soluble lens proteins and that in wet weight. Because if the total synthesis of soluble proteins were localized only in the newly formed fibers and no major shifts of water from older to newly formed fibers took place, then the concentration of soluble proteins in the newly formed fibers would be calculated to 75 to 80 percent, which appears highly improbable.

With these considerations in mind, it seems possible to give a consistent interpretation as cataractogenic factors to changes in soluble and insoluble proteins observed in tryptophane deficiency and after X-rays.

No significant changes in protein synthesis can be determined during a time interval of 10 to 12 days of tryptophane deficiency. Later on, synthesis of soluble proteins slows down sharply and significant changes can be observed already seven days later. At the same time, an almost complete inhibition of the increase in lens volume takes place.

The tryptophane-deficiency effects, therefore, have a latency period and it appears probable that during this period the tryptophane in body fluids can be maintained by mobilization of reserves at a certain level. We can, furthermore, assume that if this level drops below a certain limit the synthesis of proteins in the lens can no more proceed at the usual rate.

It seems clear that under these circumstances the epithelial cells on the surface of the lens will be in a better position to utilize the limited supply of tryptophane than the fibers in the deeper layers of the lens. Protein synthesis in the latter, therefore, predominantly is bound to be inhibited. The decrease in volume, which accompanies this decrease, will, therefore, mainly affect these deeper fibers.

As the synthesis of albuminoid in this period is not at all affected, the relative space inside the fibers occupied by the insoluble albuminoid will increase and whether this protein is localized on the cell membranes or forms a spatial network inside the fiber, the increased thickness of its layers is bound to influence the access of nutrients to the fibers and hence, the cellular metabolism.

The effects of tryptophane deficiency on the level of the protein precipitable after oxidation agrees with this interpretation. The level of this protein is related to the rate of protein synthesis in the rat lens[1] and increases sharply when the synthesis is inhibited by galactose feeding. This suggests that this protein is an intermediate in the elaboration of lens proteins and related to the so-called embryonal lens protein of François and his associates. It does not differ in its tryptophane content significantly from the rest of beta crystalline.

In the absence of sufficient amounts of tryptophane, the synthesis of this protein primarily must be slowed down and the protein already present in the fibers will be partly converted into other proteins. Its level, therefore, can be expected to decline as was indeed found in our experiments. A significant decline of this protein under the influence of tryptophane deficiency can be observed already 12 days when the decline of the total soluble protein is so small that its significance could not be ascertained.

In the case of the radiation cataract, the situation appears more complicated. At the high dose of 3,000 r, there is a latency period of about 14 days which is succeeded by a sudden decline in the rate of synthesis of soluble proteins of more than 50 percent. This period of about two weeks is followed by a stage characterized by a loss of soluble protein. It seems probable that this proteolytic breakdown of lens proteins is due to a destruction of the structure of lens fibers characteristic of mature cataracts, and it is not possible to correlate under these circumstances even early cataractic changes to protein synthesis.

At the lower dosage of 2,000 r, the course of events appears much smoother. There is a significant decrease in the synthesis of lens proteins during a time interval of 28 days and the inhibition of the rate of synthesis does not change significantly with the pro-

gressing cataractic process which develops very slowly. The volume of the lens decreases less than the net synthesis of soluble proteins, and the albuminoid formation is not affected at all. This leads to a significant decrease in the concentration of the soluble proteins in the lens.

As the effect of X-rays is mainly localized in the nuclei it seems reasonable to assume that its effect on protein synthesis will extend to the epithelium as well as to deeper layers of fibers. Cataractogenic effects of a smaller dosage of X-rays can, therefore, be ascribed to the combined effect of the increase in the thickness of the albuminoid layers and a simultaneous dilution of the intrafibrillary gel of soluble lens proteins. In this respect, the experimental X-ray cataract in its mechanism appears to resemble the galactose cataract.

Summary

1. In lenses of rats kept on tryptophane-deficient diet, the synthesis of soluble proteins is inhibited.

2. This inhibition becomes significant shortly before early cataractous changes develop.

3. The formation of albuminoid is not affected at a stage when early cataractous changes appear.

4. The ratio soluble proteins to insoluble proteins decreases before the onset of cataractous changes.

5. The protein characteristic for lenses of young rats which precipitates after oxidation, is decreased in tryptophane-deficient rats.

6. This decrease appears before any cataractous changes can be seen with the slit-lamp.

7. Lenses of five-week-old rats irradiated with 2,000 r show slowly progressing cataractous changes starting between the third and fourth week after irradition. At that time soluble proteins decrease significantly.

8. Albuminoid formation remains unchanged during the development of this form of cataract. The ratio soluble proteins to albuminoid, therefore, decreases continuously.

9. In tryptophane deficiency and X-ray cataracts, the volume of the lens decreases less than the soluble proteins. This leads to a decrease of the concentration of soluble proteins which sets in with the earliest cataractous changes.

10. A possible role of these changes in protein synthesis as a pathogenic factor in cataract formation is discussed.

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HYDROPHILIA OF THE ORBITAL CONNECTIVE TISSUE IN EXPERIMENTAL EXOPHTHALMOS*

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The most notable feature of the orbital changes in experimentally produced exophthalmos is the swelling of the connective tissue including that of the orbital fat, the septa of the glands, and of the perimysium of the muscles. It contributes to the increase in orbital contents which is the cause of exophthalmos and in the absence of the Harderian gland appears to be solely responsible for the proptosis. The histologic character of the tissue change in guinea pigs is similar to that seen in clinical exophthalmos.[1] The connective tissue fibers are separated by a fluid which infiltrates between them and between the fat cells. The edematous infiltrate contains a material precipitated by the fixative so that a homogeneous acidophilic ground substance is found throughout the orbital contents.[2] In extreme cases the connective tissue fibers, fat cells, small nerves, and vessels appear to be completely surrounded by this material. The infiltrate contains a few cells, probably of connective tissue origin, and occasionally sufficiently characteristic to be identified as lymphocytes or less often as macrophages, and, very rarely, as polymorphonuclear leukocytes.

The fluid is undoubtedly derived from the vascular system, but the cause of its appearance and retention in the tissue spaces has not been established. It is not restricted to the orbit, but is found in the connective tissue of many parts of the body.[2] An extremely attractive hypothesis offered to explain the accumulation of fluid, suggests that the connective tissue cells under abnormal conditions form an unusually large amount of hydrophilic mucopolysaccharides in the ground substance. It suggests that these connective tissue mucopolysaccharides (hyaluronic acid and chondroitin sulfate) attract and bind water which has escaped from the vascular bed.[3] This hypothesis implies that the orbital connective tissue of exophthalmic animals is more hydrophilic than that of normal animals, but no direct evidence has been brought forward to demonstrate that the exophthalmic orbital connective tissue has, in fact, an increased water binding property. Tests of the relative hydrophilia of various tissues have been conducted in a very simple, direct manner by Heringa[4] and his colleagues. Their extensive experiments are based on the theory and methods described in the classic work of Katz.[5] The experiments reported here, using this method, are intended to determine whether there is an increase in the hydrophilia of the orbital connective tissue of exophthalmic animals.

Methods

The method consists of exposing dry tissue to a moist atmosphere in a closed chamber until the avidity of the tissue components for water has been satisfied. The water taken up by the tissue was determined by repeated weighing until an equilibrium was reached between the moisture it contained and that in the atmosphere. When normal and control tissues are exposed simultaneously in the moist chamber their hydrophilia can be compared. The hydrophilia or orbital fat of normal, thyroidectomized, and exophthalmic guinea pigs, of cornea and sclera, and of normal and hypertrophied chick combs was studied by this method.

Exophthalmos was produced in 500 to 550 gm. female guinea pigs by a method which has been reported in earlier communications. The animals were completely thyroidecto-

* From the Departments of Ophthalmology and Anatomy, College of Physicians and Surgeons, Columbia University. This work was supported by a Fight-for-Sight award No. G-173 (C3) of the National Council to Combat Blindness, Inc., New York.

mized and, after a week's recovery, injected daily with 20 mg. of a crude anterior pituitary gland extract[6] plus 10 mg. cortisone for 12 days. It contained considerable quantities of all of the well known hypophyseal hormones. The thyroidectomized control animals had been operated for the same length of time as the exophthalmic animals, but had received no injections.

The tissues were removed at autopsy, placed in weighing bottles, weighed, and frozen in dry ice. They were dried by lyophilization and finally over P_2O_5 in vacuo and the dry weight determined. The weighing bottles containing the desiccated tissue were placed in a hydrator (this was a standard desiccator containing distilled water and lined with filter paper which, saturated with moisture and acting as a wick, greatly increased the water surface and aided in maintaining an atmosphere saturated with water vapor). One of each group of four or five weighing bottles contained in a beaker in the hydrator was empty, to check on condensation of moisture on the walls of the weighing bottles. Such accumulation was always minor, but a correction was made for its occurrence. The hydrator was kept at 4°C. to inhibit bacterial growth, but was returned to room temperature prior to weighing to prevent condensation of water on the cold bottles when they were removed from the hydrator. One series of experiments was conducted at room temperature; in this instance the tissues were cultured for bacterial growth at the end experiment. Orbital connective tissue from normal, thyroidectomized, and exophthalmic guinea pigs were hydrated simultaneously in each experiment so that the amount of water bound by each type of tissue could be compared under the same condtions.

Control experiments on the relative hydrophilia of cornea and sclera were conducted because the cornea contains more mucopolysaccharides than the sclera, and is more hydrophilic than that tissue.[7,8] Likewise, the hypertrophied comb of the chick contains more water and hexosamine (hyaluronic acid)[9] than does that of a normal, nonhypertrophied comb. Comb hypertrophy was induced in day-old chicks by the injection of 1.25 mg. testosterone propionate daily for 14 days. Sections of the combs of chicks thus treated showed a greatly hypertrophied metachromatic mucoid layer.

The orbital tissues were spread out on the wall of the weighing bottle as much as possible to increase the surface exposed to the atmosphere, so that the rate at which water might enter the tissue was maximal. The combs were sliced for the same reason. The cornea and sclera were not cut but were spread out in the weighing bottle. Solutions of hyaluronic acid† were also lyophilized in weighing bottles and exposed to water vapor in the same manner as were the tissues. The rate of water uptake in these experiments necessarily varied with the exposed area of the surface of the material as well as with its hydrophilia, so that sufficient time had to be allowed for the equilibrium to be attained. Tissues were kept in the hydrator for six to 38 days and extremely consistent results were obtained.

The amount of water bound by the different tissues was determined by weighing first the dry and, subsequently, the rehydrated material. The amount of water per unit dry weight

$$\left(\frac{\text{water mg.}}{\text{dry wt. mg.}} \times 100\right)$$

was plotted in the graphs shown.

Results

Under the conditions of these experiments the orbital connective tissue of the exophthalmic animals was found to be significantly more hydrophilic than that of thyroidectomized and normal guinea pigs. The difference in their affinity for water was apparent very quickly after exposing the tissue to water vapor, and a significant difference ($P < 0.01$) between exophthalmic and the

† We are indebted to Dr. Seymour P. Halbert for the hyaluronic acid used in this experiment.

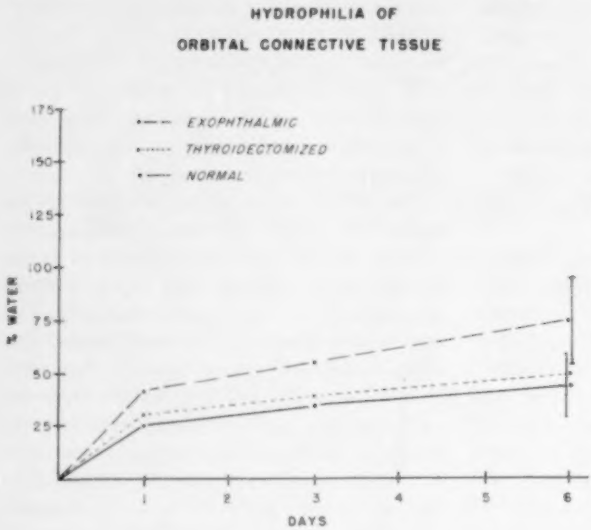

Fig. 1 (Smelser and Ozanics). Comparison of the hydrophilia of orbital connective tissue of normal, thyroidectomized, and exophthalmic guinea pigs. In all experiments hydrophilia is demonstrated by the quantity of water absorbed by the dry tissue $(\frac{\text{weight increase}}{\text{dry weight}} \times 100)$ exposed to an atmosphere saturated with water vapor at 4°C. The vertical bars represent ×2.5 the standard error of the mean. Each curve is based on data from nine animals.

normal control animals was established by the third day in the experiments (fig. 1). The slope of the curves appears to indicate that hydration was essentially complete by the sixth day. Since it could be expected that the binding of water would progress more quickly at room temperature than at 4°C., the hydrophilia of the orbital connective tissue of a second series of nine guinea pigs was tested at 22 to 24°C. It was feared that bacterial growth favored by the higher temperature might interfere with the test because of the possible destruction of hydrophilic substances. The results of this series also showed that the orbital connective tissue from exophthalmic guinea pigs was more hydrophilic than that of control animals. All preparations were made as carefully as possible to avoid bacterial contamination, but evidence of moderate numbers of airborne organisms was found by culturing at the termination of the experiment.‡ The degree of the contamination was very variable and could not be correlated with the amount of water attracted by the tissue. It was concluded, therefore, that possible bacterial contamination of the specimens was not a limiting factor in these experiments. A third comparison of the hydrophilia of control and exophthalmic orbital tissues was conducted at 4°C., in which the specimens were exposed to an atmosphere saturated with water vapor for 38 days. In this experiment also the exophthalmic orbital connective tissue was demonstrably more hydrophilic than that from normal or thyroidectomized animals.

Control experiments in which corneal and scleral tissue were treated in the same manner as were the orbital connective tissues were carried out simultaneously. Figure 2 shows that the corneas imbibed more water per unit dry weight than did the scleras from the same eyes. In each pair the cornea proved more hydrophilic than the sclera.

Comparison of the hydrophilia of normal and hypertrophied chick combs provided a second pair of control tissue. As in the orbital fat, hormonal action modified the young comb connective tissue so that it became swollen and large amounts of water were held in it. In the hydrophilia test the dried hypertrophied combs imbibed significantly greater amounts of water than did the normal comb tissue (fig. 3). This demonstrated that the tissue

‡ Our thanks are due to Mrs. Dvorah Friedman for the bacteriologic study.

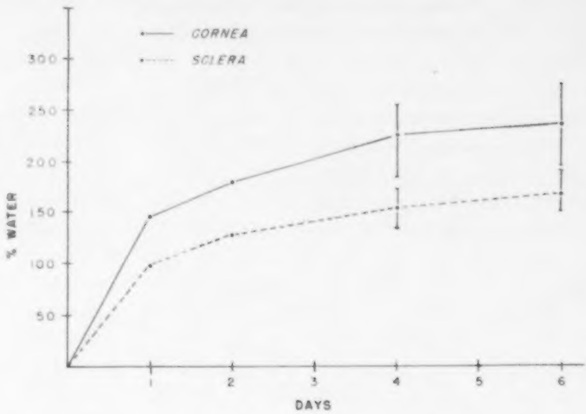

Fig. 2 (Smelser and Ozanics). Comparison of the hydrophilia of dry guinea pig cornea and sclera. The dry tissue was exposed to an atmosphere saturated with water vapor at 4°C. The vertical bars represent the maximum and minimum values obtained. Each curve is based on data from four animals.

known to contain more water and mucopolysaccharide in the living state was also more hydrophilic under the conditions of the present experiment. The hydrophilic property of hyaluronic acid under these conditions was tested simultaneously. Its hydration curve is also shown in Figure 3.

Discussion

The difference in the amount of water picked up and held by the dry orbital fat of exophthalmic and nonexophthalmic animals in these experiments is believed to be due to the hydrophilic property of the that tissue, rather than to other factors which would affect the rate of water absorption. Certainly the surface area of the different specimens exposed to water vapor varied, but the reaction was allowed to reach an essential equilibrium. The experiment in which the tissues were exposed to water vapor for 28 days showed no difference in the results from those experiments conducted for six days, or the one in which equilibrium was reached at room temperature. In the latter case the speed of absorption of water would be greater due to the higher concentration of water molecules in the atmosphere over the tissue. This experiment also demonstrated that contamination of the tissue specimens did not play

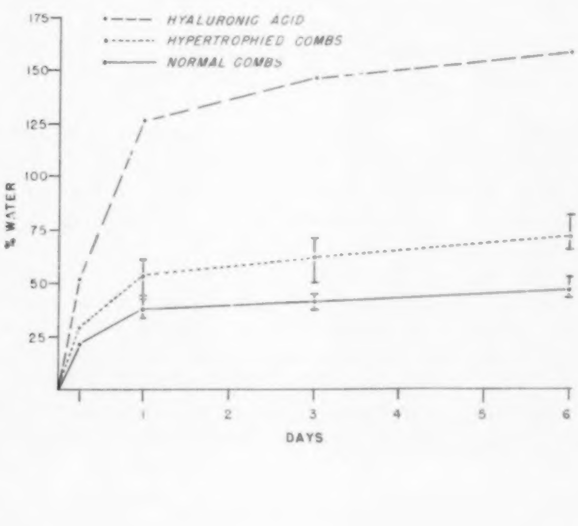

Fig. 3 (Smelser and Ozanics). Comparison of the hydrophilia of dry normal and hypertrophied chick combs. The hypertrophied combs contain more mucins and, in life, more water than do the normals. The hydrophilia of a preparation of streptococcal hyaluronic acid is shown. The vertical bars indicate the maximum and minimum values obtained.

WATER BINDING BY EXOPHTHALMIC
ORBITAL CONNECTIVE TISSUE

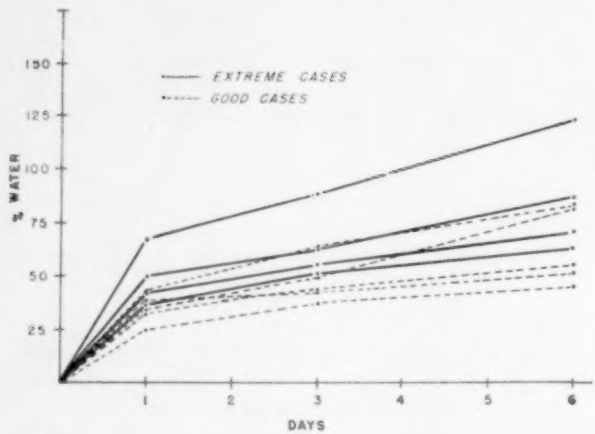

Fig. 4 (Smelser and Ozanics). Comparison of the hydrophilia of dry guinea pig orbital connective tissue of five definite and four extreme cases of exophthalmos.

a limiting role since bacterial growth, apparently of little importance at 24°C., may be expected to be of still less significance at 4°C. One experiment was made in which the hydrophilia of defatted orbital tissue was compared with that in which the lipids had not been extracted with ether because it was thought that layers of fat cells might impede the movement of water into the thin spread of dry connective tissue. However, no difference was found between the water uptake of the defatted connective tissue and that with a normal lipid content.

The importance of tissue hydrophilia in the swelling of orbital fat is particularly emphasized in Figure 4. Not only was the orbital tissue of exophthalmic guinea pigs found to take up more water than that from control animals, but orbital tissue from the most extreme cases of exophthalmos was demonstrably more hydrophilic than that from cases with moderate exophthalmos. The five most exophthalmic animals were selected on the basis of edematous hypertrophy of the orbital fat and on the degree of exophthalmos, measured at autopsy.

Although there seems to be no doubt that increased hydrophilia is an important factor in the orbital swelling, it may not be the only one. The cellular infiltration observed in the orbital tissue of clinical and experimental cases of exophthalmos suggests the presence of an inflammatory reaction which may include an increase in vascular permeability. If true, this would make water more available to the connective tissue. Such changes in permeability have, however, not been demonstrated.

The experiments reported here lead us to conclude that at least part of the swelling of the orbital connective tissue in exophthalmos is due to a change in its ability to attract and hold water. A component of the connective tissue ground substance, hyaluronic acid, is such a hydrophilic substance; it attracts and holds water under the conditions of our experiments as did the hypertrophied chick comb and orbital fat from exophthalmic animals. (figs. 1 and 3).

The swelling of the orbital connective tissue in exophthalmos could, therefore, be due to an increase in hyaluronic acid or other of the connective tissue mucopolysaccharides, as suggested by Ludwig, et al.[3] Their hypothesis postulates that the endocrine state which exists in exophthalmos causes some

connective tissue cells to secrete abnormally large quantities of mucopolysaccharide-rich ground substance. In evidence of this, they found that the hexosamine content of the orbital tissues was increased. It was thought that the additional hexosamine found in their analyses was derived from hyaluronic acid.

The edematous infiltrate of the orbital fat in exophthalmos stains metachromatically, which is in agreement with the conclusion that abnormal amounts of some mucopolysaccharide were present. It is difficult to differentiate quantitatively nonexophthalmic and exophthalmic thyroidectomized animals on this basis because the orbital tissues are metachromatic in both cases. There is not complete agreement as to the source of hyaluronic acid and other connective tissue mucopolysaccharides. Asboe-Hansen[10] has found edivence which leads his school to the opinion that mast cells are the source of this substance.

Many mast cells are found in both normal and exophthalmic orbital connective tissue. Asboe-Hansen and Iverson report[11] that the number of mast cells (determined apparently by inspection of histologic sections) is increased in the orbital fat of exophthalmic animals.

Wegelius[12] has studied the orbital connective tissue of animals treated with radioactive sulfate, and has shown in radioautographs that the orbital connective tissue of exophthalmic animals contains an increased amount of this isotope which, no doubt, was present there in the form of chondroitin sulfate.

These various studies provide evidence that the ground substance is increased in the orbital connective tissue of exophthalmic guinea pigs. Although the results of the present investigation fit this concept well, some unresolved questions remain.

The production of exophthalmos in guinea pigs is greatly facilitated by the concomitant administration of adrenal steriod hormones.[13–15] Injection of cortisone, for example, enhances the degree of exophthalmos produced, the amount of edema, and the percentage of cases which become exophthalmic as a result of pituitary extract injection. However, it has been shown in various laboratories[16–18] that cortisone reduces the amount of mucopolysaccharide present in tissues. The quantity of mast cells in mast cell tumors has also been reported to be reduced by cortisone,[19,20] and the number of mast cells in normal tissues was found to decrease and many of them lost their granules when treated with this adrenal steroid.[18] Cortisone causes a decrease in the amount of radioactive sulfate present in skin and sclera, which has been taken as evidence that mucopolysaccharides such as chondroitin sulfate are reduced[21]

The fact that cortisone has an enhancing effect on exophthalmos, and yet has a reportedly depressing action on the connective tissues ground substance, presents an unexplained paradox which sould be resolved. The present experiments do not provide any explanation of the cause of the hydrophilia which is shown, nor suggest the nature of the hydrophilic substance, but do offer a reasonable explanation of the swelling and hydration of orbital connective tissue which occurs in exophthalmos.

Summary

1. Comparisons were made of the hydrophilia of several connective tissues by exposing the dry tissues in a moist atmosphere and determining the quantity of water they attracted per unit dry weight. The amount of water held by different tissues exposed simultaneously to water vapor in a closed chamber was accepted as an indication of their relative hydrophilia. The uptake of water was allowed to proceed until an essential equilibrium had been reached.

2. This method showed that hypertrophied chick combs which in life contain more water and connective tissue mucopolysaccharides than normal nonhypertrophied combs are also more hydrophilic.

3. The orbital connective tissue of exoph-

thalmic guinea pigs was found to be significantly more hydrophilic than that of normal or nonexophthalmic throidectomized animals.

4. The degree of hydrophilia of the orbital tissue was greatest in the most severe cases of exophthalmos.

5. The present study is in agreement with the concept that the quantity of orbital connective tissue mucopolysaccharides is increased in exophthalmos. Neither the specific nature of the hydrophilic substance nor the cause of its production has been demonstrated. However, it has been shown that the orbital connective tissue of guinea pigs in which exophthalmos has been established is more hydrophilic than normally, which can account for the swelling and edema of the orbital contents.

630 West 168th Street (32).

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Discussion

Dr. David M. Maurice (San Francicso): It is always a pleasure to listen to Dr. Smelser's lucid and logical expositions. However, I was alarmed when I was asked to discuss this paper, because the subject is unfamiliar to me. I am not entitled to have much of an opinion about it. I must leave its

significance to those who are better qualified to judge. Nevertheless, I am always glad to hear of a physicochemical rather than a biochemical explanation of any such phenomena, and I tend to believe that it is true.

Dr. Smelser, there is one point on which I was rather doubtful when I read the paper, and that is the question of how valid is the method of comparing these swelling curves on the basis of dry weight. It seems to me (and I haven't read Katz's original paper) that the tissue on the hydrated side must contain a great deal of salt as well as water, and therefore this tissue, when dry, contains a greater proportion of salt. One would expect the dry tissue on the hydrated side to swell rather more than the normal side on this basis alone. The exact significance of this, and how important it is in these experiments, will become more clear when Dr. Smelser publishes his tables. I would like to ask him to discuss how significant he thinks this is.

Dr. George K. Smelser (New York): I very sincerely appreciate Dr. Maurice's discussion. We were reluctant to commit ourselves relative to the hydrophilic substance in the connective tissue or its source. The hydrophilia of the exophthalmic orbital connective tissue is greater than that of the nonexophthalmic controls, however, it is not proportional to the amount of water contained in the tissue in the living state. This can best be shown in the following table (table 1).

TABLE 1

Averaged data on the orbital connective tissues of 27 animals

The graph in Figure 1 is based on this data

Mg.	Normal	Thyroidectomized	Exophthalmic
Fresh weight	109.8	119.8	295.7
Dry weight	43.6	41.3	48.9
Water content	66.2	78.5	246.7
Mg. water per 100 mg. dry tissue of the fresh weight at autopsy	151.8	190.0	504.0
Mg. water absorbed by 100 mg. dry tissue in 6 days in moist atmosphere	43.8	52.0	75.3

THE EFFECT OF METHYLENE BLUE AND CERTAIN OTHER DYES ON CATION TRANSPORT AND HYDRATION OF THE RABBIT LENS*

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It has been clearly established that the sodium and potassium within the lens exchanges with that of the extralenticular medium and that the normal cation balance is maintained by the active transport of sodium out of the lens and potassium into it.[1-3] Evidence supports the view that this transport occurs mainly across some surface lying at or near the lens capsule and that active transport processes across the fiber or epithelial surfaces are probably of secondary importance in the maintenance of the usual cation content.[4]

Under normal circumstances, the cation balance determines, in large measure, the lenticular hydration. Where the active transport of cations is blocked by any of a variety of means, hydration invariably increases due to Donnan swelling.[5] There is no necessity to postulate a primary transport of water from the lens.

Two experimental manipulations, with ostensibly quite different effects, alter the normal lenticular cation balance. First, the interruption of energy production by enzyme poisons, by chilling, or by deprivation of oxygen or other necessary metabolites, permits the cation distribution to approach a true equilibrium with the external environment, with the lens becoming intumescent. Such effects of impaired metabolic function are reasonable since continuous energy production is essential for active transport. Second, the cation balance may be disturbed under circumstances not associated with a reduc-

* From the John E. Weeks Memorial Laboratory, Department of Ophthalmology, University of Oregon Medical School. Supported by Grant No. B187 of the National Institute of Neurological Disease and Blindness, National Institutes of Health, Bethesda 14, Maryland.

tion in available energy. Such situations include depletion of calcium from the nutrient medium, slight trauma to the lens surface, application of ATP to the external membrane and other circumstances. These maneuvers possibly alter surface organization rather than energy production, and thus may influence the mechanics of cation transport or increase the permeability of the limiting barrier.

The present studies are concerned with a group of dyes whose effects may belong more to the latter than to the former group. Study of these dyes was initiated as a result of the observation that an acid mucopolysaccharide injected into the vitreous often produced cataracts.[6] The fact that this acid mucopolysaccharide stained metachromatically with methylene blue and toluidine blue suggested the importance of studying combinations of the acid mucopolysaccharide and the dyes. The current report is concerned with a study of the dyes alone.

Other workers have suggested that these dyes may have a profound effect upon cation balance and hydration of ocular tissue. Philpot[7] observed a marked increase in corneal hydration when certain dyes were injected into the intact eye and Constant[8] found a number of dyes, including methylene blue, to cause a vacuolization of the lens, in vitro.

Methods

The temperature-reversible shift, a method which has proved useful in the past, was employed as a measure of cation transport. This technique has been previously described[9] and is only briefly reviewed here. Fresh rabbit lenses were extracted by turning back the posterior sclera and cutting the zonules, care being taken to avoid touching the lens. The lens was immersed on its posterior surface in three ml. of a balanced salt medium comparable in ionic composition to aqueous humor. Dyes or other material in the indicated amounts were added. Unless otherwise specified, the tube was flushed with a mixture of 95-percent oxygen and five-percent carbon dioxide, tightly stoppered and refrigerated at 0°C. After 40 hours, one of the pair of lenses was removed for analysis while the other was placed in an incubator at 37°C. for an additional six hours. For analysis, the lenses were dried to constant weight at 105°C., ashed, and the sodium and potassium determined on a Baird Associates flame photometer. In a few instances, lenses were incubated at 37°C. for a period of 24 hours without prior cooling.

Glucose utilization was measured in lenses incubated for six hours in a solution containing 90 mg. percent glucose. At the end of this period, the glucose concentration of two samples of the medium, one incubated with and the other without a lens, was determined. The difference represented glucose utilization. Under the conditions of the experiment the value can be taken as a measure of the metabolic incorporation of glucose, the diffusion of unmetabolized glucose to or from the lens, being insignificant. The method of Somogyi was employed for glucose determinations.[10]

Results

Addition of methylene blue in sufficient concentration had a profound effect upon the ability of the lens to recover from the cold-induced cation shift (table 1). In a concentration of 5×10^{-5} M, methylene blue blocked completely the excretion of sodium from and the accumulation of potassium within the lens. Partial blockade was observed in concentrations of 2.5×10^{-5} M, whereas the influence of 1×10^{-5} M was minimal. Concentrations higher than 5×10^{-5} M caused the most marked increase in hydration. This is attributable to the greater cation accumulation in the lens.

A more profound potassium loss and sodium gain were noted during refrigeration when higher concentrations of the dye were used. This may reflect an increase in permeability of the limiting barrier rather than a different steady-state. Similar observations have been made on the erythrocyte. How-

TABLE 1

EFFECT OF METHYLENE BLUE ON CATION TRANSPORT AND WATER CONTENT OF RABBIT LENSES

Concentration of Methylene Blue	Procedure*	No. of Lenses	Potassium meq./1000 gm. Water	Sodium meq./1000 gm. Water	% Recovery		Water %	Total Base	
					K^+	Na^+		meq./1000 gm. Water	meq./100 gm. Dry Wt.
0.0	1	68	88.8±15.4	59.2±15.4			66.8±1.0	148.0	29.8
	2	69	125.1± 7.7	24.3± 5.2	104.9	94.6	66.0±1.0	149.4	29.0
1×10^{-6} M	1	10	90.6± 9.0	58.3± 9.9			66.5±0.3	148.9	29.5
	2	11	118.4±14.2	27.5±10.5	84.8	85.6	66.7±1.2	145.9	29.2
2.5×10^{-6} M	1	15	85.8±14.1	62.7±13.5			67.1±1.0	148.5	30.3
	2	15	100.7±13.6	48.0±15.0	39.6	36.4	67.9±1.2	148.7	31.4
5×10^{-6} M	1	19	63.7±26.8	88.5±34.0			67.6±3.0	152.2	31.8
	2	18	50.8±23.5	103.2±26.7	<0.0	<0.0	69.1±3.0	154.0	34.4
5×10^{-4} M	1	10	41.2±14.1	115.1±22.8			68.1±3.0	156.3	33.4
	2	10	26.2± 4.2	135.0± 6.1	<0.0	<0.0	70.8±1.2	161.2	39.1
5×10^{-3} M	1	10	33.2±10.9	129.4±14.3			70.6±2.3	162.6	39.0
	2	9	28.2± 9.0	135.1±11.2	<0.0	<0.0	72.2±1.9	163.3	42.4

* 1. Indicates refrigeration of lenses from 40–46 hours at 0°C.
2. Indicates refrigeration of lenses from 40–46 hours at 0°C. followed by incubation at 37°C. for 6 hours.

ever, the loss of potassium noted following exposure of the red cell to methylene blue, has been attributed to an increased rate of cation diffusion out of the cell rather than to a decreased rate of accumulation.[11] While it is not possible to accurately measure the two rates with the technique we have employed our data indicate that in all effective concentrations of methylene blue, active transport is reduced while only in higher concentrations is the rate of diffusion along a concentration gradient increased.

Methylene blue is a basic dye whose charge might influence cation transport. Another organic cation, physostigmine, reduces the accumulation of potassium by the erythrocyte presumably because of its positive charge.[12] (Others have thought that the anticholinesterase activity of physostigmine is responsible for the potassium loss it induces in red cells.[11,13] For reasons which need not be considered here, however, this influence is probably minimal.[14] To test the possibility that the inhibitory action of methylene blue resulted from its positive charge, the effect of acid fuchsin, basic fuchsin, and physostigmine on cation transport was studied (table 2). None of these substances exerted marked effects on cation recovery following refrigeration at 0°C. but basic fuchsin had an observable effect in a concentration twice that of methylene blue. The effect of physostigmine was minimal in the concentration employed.

Methylene blue stands about midway in the oxidation-reduction potential scale and as such can serve as an hydrogen or electron acceptor being subsequently oxidized by molecular oxygen. The redox potential of the medium could conceivably alter a cation transport which required the movement of electrons across a barrier of molecular dimensions, much as suggested, for example by Conway.[15] Indeed, Conway and Kernan[16] have shown that, in the yeast cell, both the potassium and the sodium pump are influenced by the oxidation-reduction potential of the external medium. They further demonstrated that the E_o value of an added dye gives a fair approximation of its relative effect on the oxidation-reduction potential of cultures of yeast cells.

We have tested the effect of dyes with varying redox potentials on cation transport of the lens. In a range of E_o values (at pH = 7.0) from that of toluidine blue (+ 115 millivolts) to neutral red (− 325 millivolts) all dyes employed completely blocked cation transport (table 3). In the presence of toluidine blue and methylene blue the cation exchange during refrigeration was greater than normally observed,

TABLE 2

EFFECT OF CHARGE OF DYE ON CATION TRANSPORT AND HYDRATION OF RABBIT LENSES

Additive	Procedure*	No. of Lenses	Potassium meq./1000 gm. Water	Sodium meq./1000 gm. Water	% Recovery		Water %	Total Base	
					K^+	Na^+		meq./1000 gm. Water	meq./100 gm. Dry Wt.
1×10^{-4} M acid fuchsin	1	11	81.7 ± 10.0	63.2 ± 9.4			67.8 ± 0.8	144.9	30.5
	2	11	120.6 ± 9.2	24.9 ± 7.6	93.3	93.6	67.2 ± 0.6	145.5	29.8
1×10^{-4} M basic fuchsin	1	13	87.1 ± 7.1	58.7 ± 8.2			68.2 ± 0.8	145.8	31.3
	2	14	108.4 ± 10.5	36.0 ± 9.6	58.7	62.4	68.4 ± 1.1	144.4	31.3
5×10^{-5} M physostigmine	1	10	78.6 ± 15.0	65.1 ± 10.0			67.1 ± 1.0	143.7	29.3
	2	10	111.4 ± 6.0	32.9 ± 5.6	73.2	75.2	67.3 ± 1.5	144.3	29.7

* 1. Indicates refrigeration of lenses from 40–46 hours at 0°C.
2. Indicates refrigeration of lenses from 40–46 hours at 0°C. followed by incubation at 37°C. for 6 hours.

again suggesting an increased permeability of the barrier. As in other instances, greater lenticular hydration was associated with more marked cation accumulation.

An unexpected effect of neutral red was observed during the course of these experiments. Neutral red is relatively impure and quite insoluble in water but more readily soluble in alcohol. In a series of experiments conducted to be certain of complete solution, the dye was dissolved in a small amount of alcohol before dilution with the incubation medium. Control studies employing the same amount of alcohol were also done. When dissolved in alcohol the inhibitory effect of neutral red on cation transport was markedly reduced. The alcohol itself did not alter the usual recovery nor did it alter the absorption spectrum of the dye. The exact reason for this effect of alcohol is not yet certain. The explanations which suggest themselves are now being explored.

The dyes mentioned above are all uncoupling agents with varying degrees of effectivity.[17] It is logical to consider that their effect on cation transfer might result from depletion of the supply of high energy phosphate. Dinitrophenol is another potent uncoupling agent.[18] Previous studies of the effect of this compound on excised lenses led us to conclude that, in concentrations which did not reduce glucose utilization, this potent uncoupling agent had minimal effect on cation transport.[4] Some of these data, part of which have been published, are listed in Table 4. It seems likely that higher concentrations of dinitrophenol than of methylene blue are required to completely block cation transport although further studies will be necessary before this can be stated with

TABLE 3

EFFECT OF DYES OF VARIOUS OXIDATION-REDUCTION POTENTIALS ON CATION TRANSPORT AND WATER BALANCE OF RABBIT LENSES

Additive	Procedure*	No. of Lenses	Potassium meq./1000 gm. Water	Sodium meq./1000 gm. Water	% Recovery		Water %	Total Base	
					K^+	Na^+		meq./1000 gm. Water	meq./100 gm. Dry Wt.
5×10^{-6} M toluidine blue	1	10	54.2 ± 24.6	103.6 ± 31.0			68.9 ± 2.5	157.8	35.0
	2	8	44.2 ± 12.4	116.8 ± 15.6	<0.0	<0.0	70.1 ± 1.6	161.0	37.7
5×10^{-6} M brilliant cresyl blue	1	10	48.6 ± 14.3	102.6 ± 19.5			67.8 ± 2.1	151.2	31.8
	2	10	46.3 ± 18.8	106.9 ± 22.4	<0.0	<0.0	68.4 ± 2.4	153.2	33.2
5×10^{-6} M neutral red	1	9	79.9 ± 5.9	65.3 ± 7.1			67.8 ± 1.0	145.2	30.6
	2	10	78.6 ± 5.6	76.5 ± 5.8	<0.0	<0.0	68.7 ± 1.0	151.1	33.2
5×10^{-6} M neutral red dissolved in ethyl alcohol	1	10	77.1 ± 15.0	68.1 ± 6.8			67.5 ± 1.5	145.2	30.2
	2	9	110.0 ± 4.5	39.1 ± 5.3	71.1	63.3	66.7 ± 1.0	149.1	29.9
Ethyl alcohol (0.08%)	1	9	92.2 ± 6.2	56.1 ± 6.3			66.8 ± 0.6	148.3	29.8
	2	10	122.5 ± 6.0	24.9 ± 5.9	97.1	92.3	66.8 ± 0.6	147.4	29.7

* 1. Indicates refrigeration of lenses from 40–46 hours at 0°C.
2. Indicates refrigeration of lenses from 40–46 hours at 0°C. followed by incubation at 37°C. for 6 hours.

TABLE 4

EFFECT OF DINITROPHENOL ON CATION TRANSPORT AND HYDRATION OF RABBIT LENSES

Concentration of Dinitrophenol	Procedure*	No. of Lenses	Potassium meq./1000 gm. Water	Sodium meq./1000 gm. Water	% Recovery		Water %	Total Base	
					K^+	Na^+		meq./1000 gm. Water	meq./100 gm. Dry Wt.
0.0	1	68	88.8 ± 15.4	59.2 ± 15.4			66.8 ± 1.0	148.0	29.8
	2	69	125.1 ± 7.7	24.3 ± 5.2	104.9	94.6	66.0 ± 1.0	149.4	29.0
1×10^{-4} M	1	9	76.5 ± 5.8	73.0 ± 7.6			67.0 ± 0.7	149.5	30.4
	2	9	114.9 ± 4.1	29.0 ± 4.7	81.9	86.6	67.0 ± 0.4	143.9	29.2
1×10^{-3} M	1	9	75.8 ± 9.2	77.0 ± 10.9			67.8 ± 0.9	158.4	33.4
	2	9	81.4 ± 4.9	70.7 ± 5.4	11.8	11.5	68.2 ± 0.8	146.5	31.4
1×10^{-2} M	1	10	63.9 ± 7.7	80.4 ± 5.9			68.4 ± 0.9	144.3	31.2
	2	9	46.4 ± 11.1	90.9 ± 11.7	<0.0	<0.0	70.0 ± 1.4	137.3	32.0

* 1. Indicates refrigeration of lenses from 40–46 hours at 0°C.
2. Indicates refrigeration of lenses from 40–46 hours at 0°C. followed by incubation at 37°C. for 6 hours.

certainty. Dinitrophenol did not induce as marked an exchange of cations during refrigeration as did methylene blue. Whether this is a difference of degree or of kind cannot be stated.

A large accumulation of literature indicates that methylene blue and the other dyes have profound photodynamic effects. That is, the dyes alter certain biologic processes only when exposed to visible light. When light was excluded in our experiments, methylene blue in a concentration of 5×10^{-5} M did not exert the deleterious effect on cation transport seen when ambient illumination was permitted (table 5). (It should be noted that all specimens were refrigerated in the dark, exposure to light occurring only during subsequent incubation at 37°C.)

This effect of excluding light was also observed when lenses were incubated at 37°C. for a period of 24 hours without prior refrigeration (table 6). Under these circumstances, the lenses which were maintained in the dark showed the increase in potassium content which is normally observed when rabbit lenses are incubated at 37°C. in a nutrient medium. On the other hand, the lenses incubated with methylene blue in the presence of light demonstrated a variable loss of potassium, a gain of sodium, and, generally, an increase in hydration. The rather high standard deviation of the lenses incubated in light probably indicates a borderline biologic response at the concentration of methylene blue employed.

Logically, this effect of light should be blocked if leucomethylene blue were employed, since a photodynamic effect may be observed only when the absorption of radiant energy in the visible range occurs. To study

TABLE 5

STUDIES OF THE PHOTODYNAMIC EFFECT OF METHYLENE BLUE ON CATION TRANSPORT AND HYDRATION OF RABBIT LENSES

Additive	Procedure*	No. of Lenses	Potassium meq./1000 gm. Water	Sodium meq./1000 gm. Water	% Recovery		Water %	Total Base	
					K^+	Na^+		meq./1000 gm. Water	meq./100 gm. Dry Wt.
5×10^{-5} M	1	20	72.9 ± 22.5	75.3 ± 25.2			67.5 ± 2.3	148.2	30.8
Methylene blue (light excluded)	2	17	104.5 ± 17.0	43.9 ± 19.9	62.6	59.2	66.8 ± 1.4	148.4	29.8
Atmospheric control	1	7	89.6 ± 11.6	67.3 ± 8.3			66.5 ± 1.5	156.9	31.1
	2	7	121.8 ± 8.3	28.3 ± 6.2	95.3	86.7	66.9 ± 1.7	150.0	30.1
$Na_2S_2O_4$	1	14	63.3 ± 22.8	75.2 ± 20.3			70.7 ± 2.9	138.5	33.4
alone	2	13	92.8 ± 16.5	49.0 ± 11.9	49.5	49.1	69.5 ± 2.4	141.8	32.3
5×10^{-5} M	1	12	80.6 ± 10.0	57.8 ± 13.2			69.1 ± 2.3	138.4	30.9
methylene blue plus $Na_2S_2O_4$	2	13	91.9 ± 17.6	45.5 ± 16.7	34.6	26.4	68.4 ± 1.7	137.4	29.7

* 1. Indicates refrigeration of lenses from 40–46 hours at 0°C.
2. Indicates refrigeration of lenses from 40–46 hours at 0°C. followed by incubation at 37°C. for 6 hours.

TABLE 6

EFFECT OF LIGHT ON THE CATION AND WATER CONTENT OF RABBIT LENSES INCUBATED FOR 24 HOURS AT 37°C. IN A MEDIUM CONTAINING 5×10^{-6} M METHYLENE BLUE

	Procedure*	No. of Lenses	Potassium meq./1000 gm. Water	Sodium meq./1000 gm. Water	Water %	Total Base meq./1000 gm. Water	Total Base meq./100 gm. Dry Wt.
Fresh lenses	1	75	123.4 ± 3.7	22.3 ± 4.9	66.5 ± 1.3	145.7	28.9
Light not excluded	2	9	107.3 ± 27.7	60.1 ± 34.4	68.1 ± 3.0	167.4	35.7
Light excluded	2	9	134.8 ± 5.5	24.3 ± 6.3	66.1 ± 1.1	159.1	31.0

* 1. Indicates fresh lenses.
2. Indicates incubation at 37°C. for 24 hours without prior refrigeration.

this point, methylene blue was reduced to the colorless state with sodium hydrosulfite. This substance itself was somewhat toxic to the system as evidenced by the fact that, in the quantity utilized to reduce the methylene blue, cation transport was considerably reduced (table 5). However, in the presence of reduced methylene blue an appreciable cation transport was observed. (In these studies the medium was overlaid with atmospheric air instead of the 95-percent oxygen-five-percent carbon dioxide mixture since with 95-percent oxygen it was difficult to maintain methylene blue in the reduced form. Even so, at 21-percent oxygen, the preparations had to be watched quite carefully and sodium hydrosulfite added periodically to keep the methylene blue reduced. In the control situation [without methylene blue or other additive] 21-percent oxygen supported cation transport as well as the usually employed 95-percent oxygen.)

Other biologic effects of methylene blue include the stimulation of oxygen and glucose uptake.[19-21] Increased glucose utilization appears to be a direct oxidation probably via the hexose monophosphate shunt.[22] In these experiments, methylene blue stimulated glucose utilization to an equal degree in the presence or absence of light (table 7). The ability of other dyes and of dinitrophenol to stimulate glucose utilization was variable, neutral red (in alcohol), for example, having little or no effect. When a high concentration (10^{-2} M) of dinitrophenol was employed glucose utilization was depressed.

Discussion

It would be folly to understate the complexity of the effects of methylene blue. Five major biologic effects of this compound have been considered, all of which may have some, although certainly not equal, influence on the cation balance. It should be emphasized, too, that certain of these manifestations are probably interrelated and represent different measurements of the same underlying process.

The first of these is the effect of the compound as a cation. Its influence was found to be minimal at most. This is not too surprising. A common ion interference would be reasonable only if a higher concentration of the dye had been required. Any effect of methylene blue as a cholinesterase inhibitor can most likely be excluded also since physostigmine is a more potent inhibitor of this

TABLE 7

EFFECT OF VARIOUS DYES AND DINITROPHENOL ON THE GLUCOSE UTILIZATION OF RABBIT LENSES

Additive	No. of Lenses	Glucose Utilization, mg. of glucose/lens/6 hours
Control	63	1.13 ± 0.10
5×10^{-6} M methylene blue (light not excluded)	12	1.40 ± 0.09
5×10^{-6} M methylene blue (light excluded)	12	1.44 ± 0.10
5×10^{-6} M neutral red	12	1.09 ± 0.09
5×10^{-6} M brilliant cresyl blue	12	1.32 ± 0.10
1×10^{-6} M dinitrophenol	18	1.26 ± 0.09
1×10^{-2} M dinitrophenol	6	1.03 ± 0.10

enzyme but had only slight effect on the cation balance.

Second, there is no evident relation of the oxidation-reduction potential of these compounds to cation transport under the conditions of the experiment. This does not rule out the possibility that the cation pump functions by the movement of an electron along an orderly chain of oxidation-reduction enzymes at the lens surface. Modification of the redox potential of the environment would not of necessity alter this chain of events providing essential metabolites were present. Much, too, might depend on the concentration of the redox indicator. Schwartz and Leinfelder[23] demonstrated an apparent influence of the oxidation-reduction potential on cation exchange and hydration of the lens. Their results do not strictly pertain to the problem as here delineated, however, but are more concerned with the ability of the lens to utilize some hydrogen acceptor other than oxygen.

Third, methylene blue and similar dyes act as uncoupling agents and reduce the availability of high-energy phosphates, particularly ATP. This compound serves as the major source of immediate energy for many biologic processes and is considered essential for cation transport in many systems. It seems reasonable that part of the blockade of cation transport exerted by dyes such as toluidine blue, methylene blue, brilliant cresyl blue, and neutral red may be due to their effect as uncoupling agents. To conclude otherwise would be unsound at the moment. Interpretation of the effect of dinitrophenol is not certain. The mechanism of action of all uncoupling agents is apparently not the same[24] which may account for the apparent discrepancies in effective concentration between dinitrophenol and the dyes.

Fourth, the photodynamic action of methylene blue appears to be of greatest significance. Of the compounds tested here, toluidine blue, brilliant cresyl blue, methylene blue, and neutral red all have photodynamic activity while dinitrophenol does not. The biologic manifestations of photodynamic activity on various structures are multiple. Of these, photodynamic hemolysis closely resembles the effect we have measured. The lysis of red cells induced by the irradiation of a mixture of erythrocytes and an active dye is attributed in part to an increase in permeability of the cell membrane to cations.[25]

Among the known photodynamically induced chemical changes, the oxidation of proteins appears to offer a good avenue for future study. Certain amino acids, such as the cyclic structures, histidine, tryptophane, and tyrosine, as well as the sulfur-containing acids, are readily oxidized by methylene blue in the light.[26] The photodynamic alteration of various enzymes and proteins by this dye seems to result from attack on these amino acids.[27-29] Such activity may explain the effect of methylene blue on cation transport of the lens. One can only speculate on the exact mechanism by which this is mediated. The possibility of oxidation of essential enzymes certainly exists, even though the dye does not appear to be a metabolic poison. Assuming that the permeability of the barrier is a function distinct from active transport, one might consider that these dyes may increase the permeability of the limiting barriers by oxidizing a protein constituent.

The fifth metabolic effect of methylene blue studied here was its effect on glucose uptake. A marked stimulation of glucose utilization by methylene blue in particular was observed. As mentioned previously, this dye also causes stimulation of glucose utilization by the erythrocyte, the particular pathway involved seeming to be an oxidative shunt at the hexose monophosphate stage. At the present time it would be erroneous to state that this particular shunt can or cannot provide energy for cation transport. However, it can be stated that the activation of the shunt is not associated with the inhibitory activity of methylene blue on cation transport.

It seems likely that the inhibitory effects of methylene blue and the other dyes is

largely a surface phenomenon. The dyes, particularly methylene blue, stain the capsule and the most peripheral cortex quite readily but leave the large body of the lens untouched. Yet the shift in the cation balance is of such magnitude that the entire cation content of the lens must be considered to have been affected. This again demonstrates the fact that cation transport occurs across the lens capsule. Probably only by such is control of the hydration between fibers feasible.

Summary

1. The effect of various dyes and other compounds on the excretion of sodium and accumulation of potassium by the previously refrigerated rabbit lens has been determined.

2. Methylene blue, toluidine blue, brilliant cresyl blue, and neutral red blocked cation transport in a concentration of 5×10^{-5} M. Dinitrophenol had a similar effect but only in a considerably higher concentration.

3. Acid fuchsin, basic fuchsin, and physostigmine had only minimal effect on cation transport.

4. The inhibitory effect of methylene blue was partially voided by excluding light from the incubation medium or reducing the dye to the colorless state.

5. The stimulatory effect of methylene blue on glucose utilization was equally evident whether light was excluded or not.

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ADDITIONAL OBSERVATIONS ON THE BIOELECTRIC POTENTIALS OF THE LENS*

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The lens is a unique physiologic structure which has no vascular supply and which maintains transparency by a low metabolic rate.[1] It maintains an ionic composition quite different from the aqueous humor in which it is bathed.[2] The aqueous contains high Na^+, Cl^-, and low K^+ at about the concentrations found in plasma and extracellular fluid. In contrast the lens contains low Na^+, Cl^-, and high K^+ at about the concentrations found inside striated muscle fibers. The mechanism for maintaining concentration gradients between the lens and aqueous presumably is by active transport at the membrane barriers of the lens.[2,3] Electric potential differences exist across biologic membranes which maintain such concentration gradients. It is unknown which membranes of the lens are responsible for maintaining the ionic composition of the lens, that is, whether active transport occurs at the membranes of the lens fibers or at a membrane in or near the lens capsule.

Brindly,[4] using microelectrodes, reported a "resting" potential of about 70 mv. between the interior of the lens and the vitreous, negative inside, in rabbit and frog eyes. Complete replacement of Na^+ in the bathing medium by K^+ abolished the potential of the frog lens; recovery in Ringer solution was almost complete. Replacement of Cl^- by $SO_4^=$ had no effect. Brindley concluded that the potential depended upon ionic gradients across a membrane much more permeable to K^+ than to Na^+ or Cl^-; he postulated that this membrane may be either the capsule or the lens fiber membranes. However, no experiments were reported for decapsulated lenses. Brindley speculated that cataract may be due to an increase in permeability or to a decrease in the active pumping process in the mem-

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brane responsible for the potential. Other studies[5] reported on the lens potential deal mainly with the redox potential.

Löwenstein[6] hypothesized that the capsule is normally impermeable to electrolytes of the aqueous and that cataract may be produced by injury to the capsule increasing its permeability. Friedenwald,[7] using electroendosmosis, concluded that the capsule possesses a negative charge relative to a saline solution which enhances permeability to positively charged particles. Pau[8] postulated that cataract was due to an increased permeability of a functional membrane consisting of the capsule, epithelium, and superficial stroma causing exchange of electrolytes and uptake of water. Radioactive tracer studies showed that the rabbit lens was permeable to sodium.[9]

Harris and Gehrsitz[10] reported that the lens loses K^+ and gains Na^+ if the metabolic activity is lowered by cooling, metabolic poisons, or lack of glucose. They postulated an active transfer of cations across the lenticular barriers, probably the fiber membranes, and assumed that the cation shift observed in developing cataract reflected the breakdown in metabolic processes. The in vitro reversal of the lenticular cation shift induced by cold or Ca^{++} deficiency was reported.[11] Accompanying the cation shifts were increased lenticular hydration and swelling; reversal of the cation shift resulted in dehydration. Iodoacetate, fluoride, cyanide, and Ca^{++} or Mg^{++} deficiency produced a cation and water shift.[12] A "nick" to the capsule caused a rapid cation shift.

Biochemical evidence showing the capsule not to be an inert membrane has been presented by Dische and Ehrlich[13] who found glycolytic activity in bovine lens capsules from which the epithelium had been removed. They reported 0.0005M ATP in the capsules which decreased in concentration rapidly after excision due to ATPase activity; it was concluded that removal of the capsule causes severe damage to it. Aerobic metabolism was not present. Aldolase, phosphoglyceromutase, enolase, and lactic acid dehydrogenase were found in bovine lens capsules.[14] Frohman and Kinsey[15] reported that the high-energy phosphate compounds are in much greater concentration in the capsule (including the posterior capsule) and epithelium than in the cortex.

There are three possible models which may describe the electrophysiology of the lens (fig. 1):

1. The ions within the lens might be distributed uniformly, the lens fibers being freely permeable to all major ions; hence the fibers would not have transmembrane potentials. Therefore, the ionic gradients between lens and aqueous would be maintained by active transport in a membrane at or in the capsule; hence a potential difference would exist across the capsule. The transcapsular potential would be negative inside and its magnitude given by the Nernst equation

$$E = \frac{RT}{zF} \ln \frac{(K^+ \text{ in})}{(K^+ \text{out})}$$

if the potential approached the K^+ equilibrium potential, as is currently accepted for nerve and muscle. However, if the partial conductances of Na^+ and Cl^- were not negli-

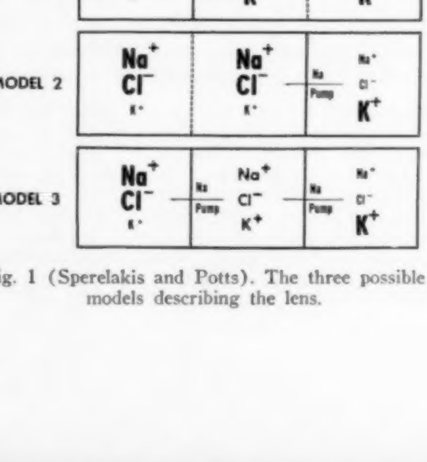

Fig. 1 (Sperelakis and Potts). The three possible models describing the lens.

gible relative to the K^+ conductance, then the Falk-Gerard equation[16] would more accurately express the potential expected:

$$V_{capsule} = \frac{g_{K^+}}{\Sigma g}\frac{RT}{zF}\ln\frac{[K^+_{in}]}{[K^+_{out}]} + \frac{g_{Na^+}}{\Sigma g}\frac{RT}{zF}\ln\frac{[Na^+_{in}]}{[Na^+_{out}]} + \frac{g_{Cl^-}}{\Sigma g}\frac{RT}{zF}\ln\frac{[Cl^-_{in}]}{[Cl^-_{out}]}$$

where g is the conductance for each ion, Σ g is the total conductance, and the other symbols have their usual connotation.[3,16] This model assumes the lens to be one giant cell with the capsule equivalent to the cell membrane.

2. The ions might not be distributed uniformly in the lens cortex, ionic gradients existing across the fiber membranes. The capsule may be freely permeable to all major ions, hence no transcapsular potential would be present. Active ion transport and transmembrane potentials, negative inside, should be present across the fiber membranes. The ionic composition of the extracellular space between fibers would be the same as the aqueous (high Na^+, Cl^-, and low K^+); the intracellular composition would be low in Na^+, Cl^- and high in K^+. Since the extracellular space of the lens is low,[9] the intracellular ionic composition would account for the over-all ionic composition of the lens. This model assumes the lens to be similar to muscle tissue.

3. Ionic gradients would exist across the individual fiber membranes and, in addition, across the lens capsule. Active ion transport and potential differences should be present at both sites. The transcapsular potential would be negative inside and in series with the fiber transmembrane potentials which would be negative inside. The magnitude of the potentials at the two sites would be dependent upon the partial conductances and ionic gradients of aqueous to extracellular fluid and extracellular fluid to intracellular fluid, assuming the activity coefficients of the ions in the various fluids to be equal. The ionic composition of the extracellular space would be intermediate between that of the aqueous and that of the intrafiber space. This model assumes the lens to consist of many individual cells within one giant cell.

To test which of these three models most accurately describes the actual conditions in the lens was the purpose of the experimentation conducted. The results obtained with the potential and current measurements lend considerable support in favor of the third model, that is, both the capsule and the fiber membranes act as electric barriers having potential differences across them directed negatively inward and probably are sites of active ion transport.

Material and Methods

Most experiments utilized bovine eyes obtained from a packing house two to six hours after removal from the animal; the eyes were stored in a moist atmosphere at 22°C. until used. One experiment was performed at the packing house in order to ascertain the value for the transcapsular potential within the five minutes after removal of the eyes from the animals. A few experiments utilized freshly removed rabbit and frog eyes; the frogs were decapitated and the rabbits were injected intravenously with Nembutal (30 mg./kg.). Some bovine and rabbit eyes were stored in isotonic NaCl at various temperatures (5°C. to 42°C.) before used. One rabbit experiment was performed in vivo with the rabbit under deep Nembutal anesthesia and the eyes and lenses left in situ. Some experiments were done on lenses left in their normal in situ position in the eyeball by reflection of the cornea and iris, thus exposing the anterior capsule of the lens; other experiments were on isolated lenses to which the suspensatory ligaments and part of the ciliary body were left attached and stored in various isosmotic solutions until measured.

The transcapsular potentials were measured by reversible Ag:AgCl agar-saline wick electrodes. The resting potentials of the lens fibers were measured by glass microelectrodes with tip diameters less than 0.5

micron and filled with 2M KCl. The impedances of the microelectrodes varied between 10 to 40 megohms, and the tip potential differences in isotonic NaCl and KCl were less than 4.0 mv. A Ag:AgCl electrode made contact with the KCl of the microelectrode, and the reference electrode was a Ag:AgCl agar-saline wick electrode. The microelectrode was mounted on a micromanipulator; a dissecting microscope and spot lamp enabled gross observation of microelectrode position.

The electrodes were connected to a stable direct-coupled preamplifier designed in this laboratory[17] which had an electrometer (Raytheon 5886) input and cathode-follower output. The electrometer input enabled a high input impedance ($> 10^{12}$ ohms) and a very low grid current ($< 10^{-12}$ amp.). The preamplifier had a calibration circuit in steps of 15±0.15 mv. The output from the preamplifier led to the DC amplifier of an oscilloscope (DuMont type 333); the potentials were measured as deflections of the oscilloscope beam zero position.

Results

I. Lens fiber transmembrane potentials recorded by microelectrodes

The resting potentials of individual lens fibers of the bovine eye were measured by microelectrodes with the lens capsule removed or the transcapsular potential short-circuited. The fibers were negative inside relative to the outside. The mean with the standard error of the mean for 26 lenses measured was −23.3±1.2 mv. (table 1). In one frog lens examined an average value of −20 mv. was obtained for the resting potential of the fibers. The fiber resting potentials (table 2) were decreased as much as 47 percent by prior treatment of the isolated lenses for approximately one hour with various agents, such as ultraviolet, isotonic KCl, and 0.0027M ethylenediamine tetraacetic acid (EDTA) (see results below).

The trace of a typical microelectrode impalement of a single lens fiber is shown in Figure 2. This fiber was deep in the cortex and had a resting potential of − 18 mv. Withdrawal or further penetration of the microelectrode by means of a micromanipulator resulted in a sharp return of the oscilloscope trace to the zero potential level. A small peculiar potential (M± standard error

TABLE 2

Fiber resting potentials of treated bovine lenses

(Lenses measured in order shown using same microelectrode)

Lens No.	Treatment (1 hr.)	Average Resting Potential (mv.)
1	Control	−25.3
2	uV (9 in.)	−15.6
3	Isot. KCl	−17.3
4	Control	−27.4
5	EDTA	−14.3
6	Control	−24.2

TABLE 1

Fiber resting potentials of normal bovine lenses

Lens No.	Average Resting Potential (mv.)	Lens No.	Average Resting Potential (mv.)	Lens No.	Average Resting Potential (mv.)
1	−16.4	10	−17.9	19	−27.4
2	−14.8	11	−18.7	20	−41.2
3	−26.1	12	−19.0	21	−36.6
4	−27.5	13	−24.2	22	−26.9
5	−22.0	14	−23.8	23	−22.8
6	−26.7	15	−19.0	24	−21.7
7	−19.0	16	−16.5	25	−22.1
8	−16.9	17	−32.5	26	−24.2
9	−17.8	18	−25.3		

Mean ±S.E.M.: −23.3±1.2 mv.

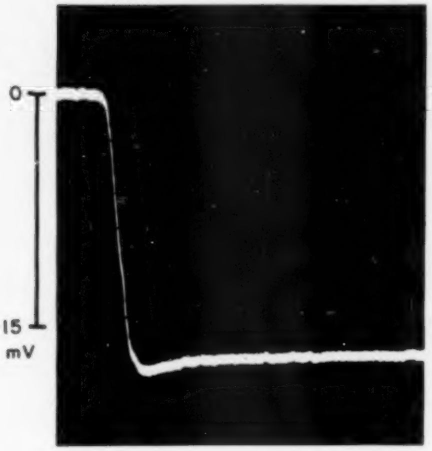

Fig. 2 (Sperelakis and Potts). Oscilloscope trace of microelectrode impalement of a single lens fiber.

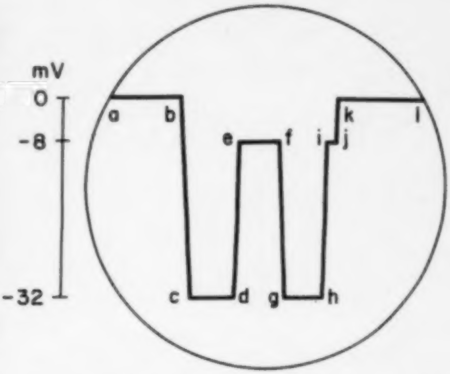

Fig. 3 (Sperelakis and Potts). Schematic diagram of microelectrode impalement of two successive lens fibers. a-b, k-l: Zero potential level, microelectrode in bathing medium. b-c: Microelectrode tip inside lens fibers, resting potential recorded. d-e: Deeper penetration resulting in passage of tip through the first cell into extracellular space (e-f, i-j); d-e distance is true resting potential. f-g: Deeper penetration of the tip resulting in impalement of the next fiber. h-i: Slight withdrawal of microelectrode. j-k: Withdrawal of tip into the medium.

of the mean of -10.3 ± 0.9 mv. relative to the bathing solution) was observed when the microelectrode tip was presumably in extracellular space of the lens, that is, not within a fiber. This potential was most evident upon withdrawal of the microelectrode following penetration of several fibers (fig. 3), hence it may be an injury potential recorded from the vicinity of injured fibers. It is unlikely that this potential is a microelectrode tip potential (Adrian[18]) since the tip potential differences between isotonic NaCl and isotonic KCl for a few microelectrodes examined were less than 3.0 mv.; rather it may be analogous to the interstitial potential reported by Bülbring[19] in pupillary smooth muscle.

II. Transcapsular potential recorded by wick electrodes

The transcapsular potentials of lenses were measured by Ag:AgCl agar-saline wick electrodes. The mean $\pm$ standard error of the mean of 55 bovine lenses measured in situ (that is, held in the normal position by the suspensory ligament) was -26.2 ± 0.6 mv., whereas the value obtained from isolated bovine lenses was -18.0 ± 0.6 mv. (table 3). The reason for this discrepancy is assumed to be due to partial injury to the capsule during the removal of the lens resulting in partial short-circuiting of the potential. In order to ascertain the effect of time, the transcapsular potentials of 12 bovine lenses were measured within five minutes after re-

TABLE 3

Transcapsular potentials of isolated bovine lenses

Experiment No.	No. of Lenses in Each Experiment	Mean ±S.E.M. (mv.)
1	10	-17.9 ± 1.0
2	13	-18.5 ± 1.0
3	18	-14.8 ± 1.0
4	8	-20.0 ± 1.3
5	3	-21.3 ± 0.4
6	6	-17.8 ± 0.8
7	4	-17.8 ± 2.0
8	4	-16.0 ± 1.3
9	3	-20.3 ± 1.0
10	4	-15.6 ± 1.5
11	7	-18.9 ± 0.4
12	3	-16.3 ± 0.7
Mean ±S.E.M.:		-18.0 ± 0.6

TABLE 4

TRANSCAPSULAR POTENTIALS OF BOVINE LENSES IN SITU MEASURED WITHIN 5 MINUTES AFTER DEATH OF THE ANIMALS

Animal No.	Lens of Pair	Transcapsular Potential (mv.)	Pair (Average) (mv.)	% Deviation of Paired Lenses from Average	% Deviation of Pair Average from Over-all Mean
1	a	−24			
	b	−24	−24.0	0.0	20.5
2	a	−33			
	b	−32	−32.5	1.5	7.6
3	a	−26			
	b	−23	−24.5	6.1	18.9
4	a	−27			
	b	−29	−28.0	3.6	7.3
5	a	−38			
	b	−38	−38.0	0.0	25.8
6	a	−38	—	—	—
7	a	−30	—	—	—

Mean ±S.E.M.: −30.2 ± 1.4 mv.

moval from the animal (table 4). The mean was −30.2 ± 1.4 mv., and the values for the pair of lenses from any one animal were much closer than lenses from different animals. Hence some physiologic factor, such as age, may be responsible for the variation in transcapsular potentials among animals of the same species.

The transcapsular potentials of eight rabbit lenses and six frog lenses were measured in situ by wick electrodes and microelectrodes respectively. In the rabbit the mean was −22.8±1.3 mv., and in the frog a mean of −49.7±4.1 mv. (at 22°C) was found. The transcapsular potential may be larger in the frog compared to the rabbit or bovine eye due to the microelectrode recording "injury" potentials from the fibers. The results with the rabbit lenses included a pair measured in vivo with the rabbit under nembutal anesthesia.

Attempts were made to isolate the anterior capsules of bovine lenses and mount them in a lucite holder and determine whether a potential difference existed across such an isolated capsule. All such experiments gave zero potential except one in which the potential rose from zero at 22°C. to −21 mv. (negative inside) at 38°C. and then returned to −3.0 mv. upon cooling. In light of the results obtained in the capsular resistance studies, it is assumed that the isolated capsule experiments gave negative results because capsular injury abolished the transcapsular resistance and short-circuited the potential.

Temperature had a marked effect upon the transcapsular potential. Each point in Figure 4 represents the average of groups of two or four lenses. There is essentially a straight-

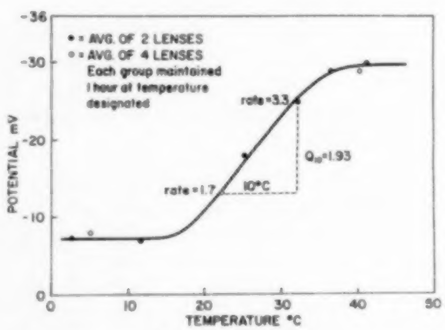

Fig. 4 (Sperelakis and Potts). Effect of temperature upon the transcapsular potential.

line rise in potential between 17°C. to 36°C. and plateaus between 2°C. to 16°C. and 37°C. to 42°C.; hence the optimum temperature is near body temperature. Assuming potential to be directly proportional to rate of reactions at a membrane and arbitrarily setting the rate at the lower plateau to 1.0, the straight-line rise portion of the curve has a Q_{10} (change in rate per 10°C. change in temperature) of 1.93, equivalent to an activation energy of −12.0 kcal/mole. Most chemical reactions have Q_{10} values of 2.0 to 3.0, whereas physical processes have Q_{10} values much lower.[20] With any one individual lens both an increased temperature (17°C. to 36°C.) and an increased period of time at this higher temperature resulted in a higher transcapsular potential. The time course of the rise in potential with time at 38°C. following preincubation at 22°C. is shown in Figure 5 for three lenses in situ; the peak potentials were reached in 16 to 26 minutes.

Various agents acted to diminish the transcapsular potential. Included among these agents are: 2:4 dinitrophenol (2:4 DNP), a high-energy phosphate uncoupler; sodium monoiodoacetate (IAA), an inhibitor of glycolysis; disodium ethylenediamine tetraacetate (EDTA), a metal chelating agent; ultraviolet irradiation (uV); and anoxia plus IAA, which blocks both aerobic and anaerobic oxidation. The concentration of these agents used is given in Table 5; the dose of uV is given as inches from a standard GE sunlamp for the time period listed. Table 5 gives the mean ± standard error of the mean for the groups treated with these agents by averaging the time periods. All the agents shown, except nitrogen (N_2) bubbling, produced a 50 percent or greater decrease in potential relative to the controls; the difference between treated and control groups is statistically significant, the p values being less than 0.01. The effect of nitrogen alone for 111 minutes was not statistically significant. Oxygen bubbling for 100 minutes had no significant effect.

Ion replacement studies on bovine lenses are complicated because complete removal of vitreous is difficult to accomplish without injury to the posterior capsule. In the present studies some vitreous was left adhering to the lenses. The effect of the presence of this vitreous was partially circumvented by mak-

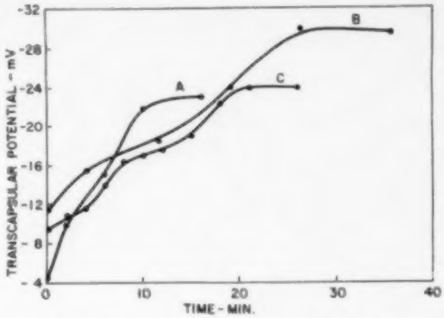

Fig. 5 (Sperelakis and Potts). Time course of rise in transcapsular potential with incubation at 38°C. after preincubation at 22°C.

TABLE 5

EFFECT OF VARIOUS AGENTS ON TRANSCAPSULAR POTENTIALS OF ISOLATED BOVINE LENSES

Agent	Average Time (min.)	Treated M ± S.E.M. (mv.)	Treated No. of Experiments	Treated No. of Lenses	Control M ± S.E.M. (mv.)	Control No. of Experiments	Control No. of Lenses	p Value
2:4DNP (0.003M.)	145	− 8.5±0.8	3	8	−17.9±1.0	3	10	0.01–0.001
IAA (0.025–0.1M.)	130	− 8.5±1.5	4	13	−18.5±1.0	4	13	0.01–0.001
EDTA (0.0028M.)	90	− 8.8±0.6	3	8	−20.0±1.3	3	8	0.01–0.001
uV (9 in.)	85	− 6.9±0.8	3	18	−14.8±1.0	3	18	0.01–0.001
N_2	111	−12.8±1.8	1	4	−17.8±2.0	1	4	0.1 –0.2
N_2+IAA (0.036M.)	24	− 8.0±0.6	1	4	−16.0±1.3	1	4	0.01–0.001

TABLE 6

EFFECT OF ION REPLACEMENT ON TRANSCAPSULAR POTENTIALS AND CLOUDINESS OF ISOLATED BOVINE LENSES

Medium (isosmotic)	Test Medium (1 hour)				Recovery Medium (NaCl, 1 hour)			
	M ±S.E.M. (mv.)	No. of Experiments	No. of Lenses	Cloudiness	M ±S.E.M. (mv.)	No. of Experiments	No. of Lenses	Cloudiness
NaCl	−16.4±1.0	6	32	0	−11.3±1.5	2	7	0
KCl	− 0.1±0.9	4	16	0	−13.2±1.1	4	16	0
Choline-Cl	−17.7±1.3	3	12	++	−10.3±1.9	1	4	0
Na_2SO_4	− 7.5±1.3	4	15	0	—	—	—	0
K_2SO_4	+ 1.9±0.4	4	15	+++	− 5.9±1.3	4	15	+ (sl.)

ing three changes with large volumes of test medium at zero, 10, and 20 minutes at 22°C. and then incubating for 40 minutes at 38°C.; a similar protocol was repeated for the recovery experiments in isotonic NaCl.

Table 6 gives the results of these studies. Isosmotic KCl (0.15M) reduced the transcapsular potential to essentially zero; recovery in isotonic NaCl was complete. Isosmotic K_2SO_4 (0.10M) caused a small reversal of sign of the potential to an average of +1.9 mv. This slightly greater effect of K_2SO_4 compared to KCl is probably due to the fact that the K^+ ion concentration in the former is 200 mEq/l while that of the latter is 150 mEq/l. Recovery after K_2SO_4 was only partially complete. Isosmotic Na_2SO_4 (0.10M) produced over a 50-percent decrease in potential; attempted recoveries in isotonic NaCl were non-conclusive, but certainly far from complete. Hence it appears that replacement of Cl^- by $SO_4^=$ (Na_2SO_4) results in irreversible partial decrease in potential. Isosmotic choline-Cl (0.15M) had no effect on the transcapsular potential.

Of the five test media, K_2SO_4 and choline-Cl produced marked clouding of the lenses, the clouding being almost completely reversed during the recovery in isotonic NaCl (table 6). Thus the choline-Cl had no effect upon the transcapsular potential but caused marked clouding, whereas KCl reduced the potential to zero but produced no clouding; hence clouding and reduced potential are separate processes.

Short-time effects of these test media on lenses left in situ were determined by dripping the test solution over the anterior capsule while the transcapsular potentials were monitored. K_2SO_4 and KCl diminished the potential to zero within six minutes; the K_2SO_4 acted about twice as rapidly as the KCl. These agents acted similarly even if added only to the periphery of the anterior capsule. Na_2SO_4 and choline-Cl had no effect; no clouding was produced by any of the test solutions during these short-time periods. The K_2SO_4 and KCl most likely act to decrease the transcapsular resistance and potential where applied and thereby short-circuit the transcapsular potential of the normal capsule areas. Further evidence for this viewpoint is presented in Table 7. In these experiments the test solutions were applied locally to only the anterior pole of the capsule by soaking small pieces of filter paper in the test solution and then applying the filter paper to the anterior capsule. The time course of the injury to the capsule in this local area was monitored by the injury potential. Of the test solutions, only K_2SO_4 and KCl produced marked capsular injury within five minutes. None of the solutions produced clouding in the test area.

III. CAPSULE: BUCKING-CURRENT EXPERIMENT, DC RESISTANCE, INJURY

Bucking-current (short-circuit) experiments, similar to those done on frog skin by Ussing and Zerahn,[21] were performed on bovine lenses in situ. A pair of current electrodes (Ag:AgCl) as well as potential meas-

TABLE 7

SUMMARY OF AGENTS PRODUCING CAPSULAR INJURY AND CLOUDING OF BOVINE LENSES IN SITU

Agent		Capsular Injury Potential (within 5 min.)	Cloudiness (within 5 min.)
Mechanical	a. Finger rub	++++	+++
	b. Forcep	++	+
Electrical (50 μamp.)	a. Cathodal	++++	+ (periph.)
	b. Anodal	0	0
Osmotic	a. Hypo-osmotic (H_2O)	0	0
	b. Hyper-osmotic		
	1. Glucose, 1M. (iso. NaCl)	+	++
	2. Sucrose, 3M. (iso. NaCl)	+	+++
	3. $MgSO_4$, 1M.	+	++
	4. NaCl, 0.5M.	++	++
	5. NaCl, 1M.	++++	++++
	6. Na_2SO_4, 1M.	++++	++++
	7. KCl, 1M.	++++	++++
	8. Choline-Cl, 1M.	++++	++++
	c. Isosmotic		
	1. NaCl	0	0
	2. KCl	++	0
	3. Choline-Cl	0	0
	4. Na_2SO_4	0	0
	5. K_2SO_4	+++	0
Chemical	a. Ether	0	0
	b. Glycerin	++	+++
	c. Versene, .003M. (iso. NaCl)	0	0

uring electrodes were used; a microammeter was in series with the current electrodes (fig. 6). Anodal current was passed through a hole in the center of the anterior capsule in steps of 5.0 μamp. and the decrease in transcapsular potential was recorded. Plots of potential versus current gave straight lines (fig. 7). Correction for the IR (potential) drops in components other than the capsule itself were obtained by removal of the entire capsule or the anterior capsule only. The intersection on the ordinate (current) of a line drawn parallel to the abscissa (potential) from the intersection of the correction line with the experimental line gives the true

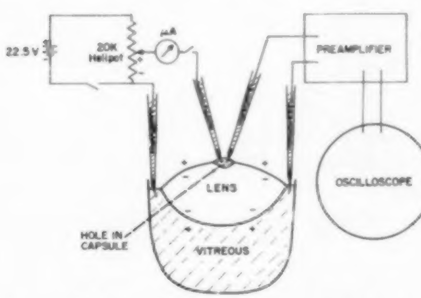

Fig. 6 (Sperelakis and Potts). Schematic diagram of the bucking-current experiment.

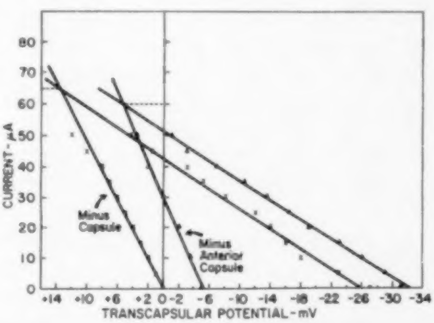

Fig. 7 (Sperelakis and Potts). Bucking-current experiments with bovine lenses in situ.

"short-circuit" current. This value averaged 62 μamp, and is equal to the maximum active transport current[21] across the posterior capsule. Since the surface area of the posterior capsule of the average adult bovine lens used was approximately 3.4 cm^2., this would be equivalent to 18 μamp./cm^2. The DC resistance of the posterior capsule can be calculated from Ohm's law ($R = E/I$) using the transcapsular potential and short-circuit current and averaged 1600 ohms/$cm.^2$ for the two experiments of Figure 7; hence the average conductance would be the reciprocal of 1600 ohms or 0.63 millimhos/cm^2. The partial conductances for the major ions were not determined.

Various treatments were found to injure the capsule by lowering the transcapsular resistance and potential. Localized areas of injury to the capsule were produced, and the extent of injury was determined as an injury potential by the use of external wick electrodes. A 100-percent injury was inferred if the injury potential was equal to the transcapsular potential determined by cutting a hole through the injured capsule area and placing the wick electrode on this hole. If the potential between the local treated area and the vitreous was zero, then a zero-percent injury was concluded. The agents tested that produced such capsular injury can be divided into mechanical, electric, hyperosmotic, ionic, and chemical. Some agents that produced capsular injury did not produce clouding of the cortical fibers in the treated area, other agents produced marked clouding but little or no capsular injury, and the remainder caused both capsular injury and clouding (table 7). The magnitude of clouding was determined qualitatively by visual inspection of the contrast between the normal and treated areas, and arbitrarily rated between zero and 4+ (4+ being the amount of clouding produced by 1M NaCl).

Mechanical injury to the center of the anterior capsule was produced within two minutes by grasping with forceps or by rubbing with an ungloved finger. These procedures produced clouding as well as capsular injury; the finger massage produced more clouding, perhaps due to chemicals present on the skin (table 7).

Injury to the anterior pole was produced by passing 50 μamp. of electric current for several minutes with the second current electrode in the vitreous. With such an electrode geometry the current density was much higher through the capsule at the electrode on the anterior capsule, hence injury was produced only at the localized area of the anterior capsule. It was found that cathodal current, but not anodal current, produced 50-percent capsular injury within three minutes and 100-percent injury at four to eight minutes (fig. 8, table 7). Hence injury was produced only when the current electrode on the anterior pole was the cathode, that is, in opposite polarity to the transcapsular potential which is negative inside. Excitation of nerve or muscle occurs with cathodal current which also is in opposite polarity to the resting

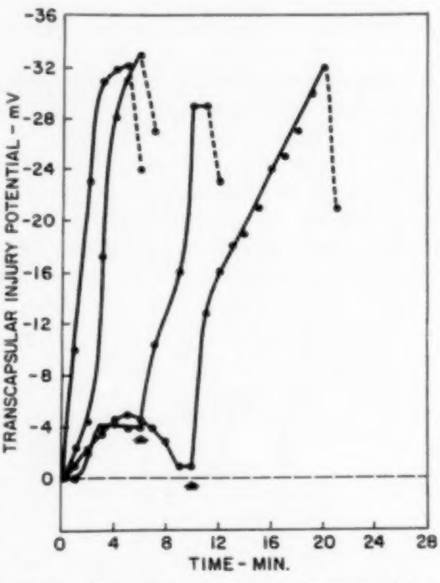

Fig. 8 (Sperelakis and Potts). Capsular injury produced by 50 μamp. cathodal current. Arrows indicate change from anodal to cathodal current; dotted lines indicate the transcapsular potential.

potential. A small amount of clouding was noted around the periphery of the anterior capsule but not at the region of the cathode. A curious phenomenon noted was that the injury potential averaged 7.0 mv. greater than the transcapsular potential, that is, the apparent injury was over 100 percent (fig. 8). If the electrode was placed on the reflected capsular flap then a potential was obtained about equal to the original injury potential; the explanation for this is unknown, but may be due to an accumulation of anions in the capsule at the injured region.

Various hyperosmotic solutions and isosmotic KCl and K_2SO_4 produced injury to the anterior or posterior capsule when applied to a local area of the capsule on filter paper (table 7). One molar or higher concentrations of NaCl, KCl, Na_2SO_4, and choline-Cl produced 4+ (maximum) injury and 4+ clouding within 30 seconds. The lowest concentration of NaCl which produced over 75-percent injury within five minutes was 0.5M. Sucrose at 3M (in isotonic NaCl), 1M glucose (in isotonic NaCl), and 1M $MgSO_4$ produced only slight (1+) injury but caused 2+ or 3+ clouding. Isosmotic KCl produced 2+ injury but not a trace of clouding, and isosmotic K_2SO_4 produced 3+ injury but no clouding within the five minutes. Isosmotic Na_2SO_4 and choline-Cl had no effect within the five minutes. Distilled water (hypo-osmotic) produced neither injury nor clouding; similarly, ether and 0.003M EDTA (in isotonic NaCl) had no effect. Glycerin rapidly produced 3+ clouding with about 2+ injury.

Discussion

The results present evidence in favor of the third model, that is, there are electric potential differences across the individual lens fiber membranes and across the lens capsule. Some evidence has been presented with the temperature and drug studies indicating that these bioelectric potentials are dependent upon metabolic energy. It can be inferred that the fiber membranes and capsule have active metabolic ion pumping mechanisms maintaining electrochemical gradients across them.

From the fiber resting potential data, it can be concluded that the ionic content of the lens is not uniformly distributed, but rather that ionic concentration gradients exist between the intracellular space and the extracellular space. Since the fiber resting potentials are negative inside and since the chloride and sodium content of the lens are low relative to potassium, it can be assumed that the K^+ concentration is higher and the Na^+ and Cl^- concentrations lower intracellularly than extracellularly. If the K^+ partial conductance were assumed to be large while those of Na^+ and Cl^- were negligibly small, then the Nernst equation gives a value of 2.5 for the ratio of intracellular to extracellular K^+, using the mean fiber resting potential of −23 mv. Further, if the extracellular space were taken to be the value for the chloride space of 8.7 percent and the K^+ concentration of the lens taken to be 125 mEq/l,[9] then the K^+ concentration of the extracellular space would calculate to be 52.7 mEq/l and that for the intracellular space 131.8 mEq/l to give a ratio of 2.5 for $(K^+in)/(K^+out)$.

If the K^+ concentration of the aqueous be taken as 4.9 mEq/l,[4] and of the K^+ partial conductance at the capsule be assumed to be unity, then the transcapsular potential would calculate to be −59.2 mv., assuming the activity coefficient of the K^+ inside the lens is equal to that in the aqueous. Since the calculated transcapsular potential is much larger than the mean observed value of 30 mv., the ratio of the K^+ partial conductance to the total conductance at the capsule and/or at the fibers must be significantly less than unity. From the ion replacement data it appears that Na^+ does not play a major role in the transcapsular potential, that is, the Na^+ partial conductance is negligibly small. Hence there is a possibility that Cl^- contributes to the transcapsular potential. Attempts were made to measure the capacitance of the capsule; the results were inconclusive,

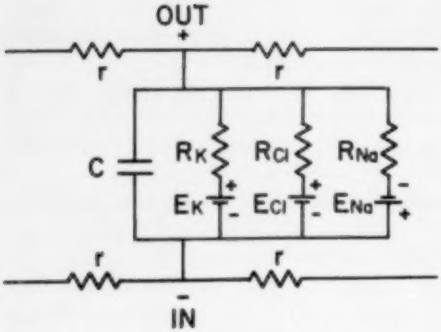

Fig. 9 (Sperelakis and Potts). Electrical equivalent circuit of the lens capsule.

but values were obtained in the range of 0.05 to 1.0 μfarad/cm.[2] An electric equivalent circuit may be drawn for the capsule and fibers in accordance with the Hodgkin-Huxley[22] circuit for nerve membranes (fig. 9).

The presence of a significant transcapsular potential is curious in view of the fact that the capsule had been generally regarded as an inert, structureless membrane and in view of the fact that only the anterior capsule has an epithelium associated with it. Experimentally it was found that removal of most of the anterior capsule with the underlying epithelium had no effect upon the posterior transcapsular potential, hence eliminating the epithelium as the source of the battery. Brindley's experiments[4] support this idea. The capsule has a large impedance of about 1600 ohms/cm.[2] which can be reduced essentially to zero in local areas by mechanical injury, hyperosmotic solutions of univalent ions, and cathodal current. This injury phenomenon enables the transcapsular potential to be observed as an injury potential using two external wick electrodes, much as the injury potential obtained from nerve or muscle. Associated with some of these local injured areas on the capsule were opacities of the superficial cortical fibers underneath the injured area. However, there is no direct relationship between capsular injury and immediate opacity since some capsular injuries occurred with no opacities and some agents produced opacities with little or no capsular injury.

The transcapsular short-circuit current was determined to be about 18 μamp/cm.[2], which is theoretically equal to the maximum active transport current. This calculates to be a movement of 115×10^{-11} equivalents of cation across the capsule per second if the surface area of the average bovine capsule be taken as 6.4 cm.[2].* (1 ampere = 1 coulomb/sec.; 1 farady = 96,500 coulombs; 1 farady = 1 equivalent of ions moved.) Assuming the cation moving outward across the capsule to be the univalent Na^+ ion due to an active Na^+ pump, this is equal to 115×10^{-11} moles of Na^+ per second. If a bovine lens be estimated to contain a total of about 0.1 mEq. of Na^+, the above calculated Na^+ movement per second during maximum operation of the pump would be about 0.000115 of the total Na^+ content of the lens at equilibrium. The value for Na^+ transport is in the order of magnitude of that described by Harris, et al., [11, fig. 2] who found about 200 mEq./1,000 gm.H_2O of Na^+ pumped out in about 10^4 sec. from rabbit lens which was previously refrigerated. Assuming the rabbit lens to contain roughly 1.5 cc. H_2O, this calculates to be about 270 by 10^{-11} equiv. Na^+/sec.

Because of its ectodermal origin and embryonic invagination the lens may be considered analogous to inside-out epidermis. Thus, considering the classical frog skin preparation, the outside of frog skin is equivalent to the inside of the lens embryologically. Furthermore, the outside of frog skin is negative while the inside of the lens is negative. Ussing and Zerahn[21] have clearly demonstrated active transport of Na^+ from outside to inside of short-circuited frog skin. The biologically-active portion of the lens capsule may be considered as being equivalent to the basement membrane of frog

* 1 ampere = 1 coulomb/sec.
1 farady = 96,500 coulombs = 1 equivalent of ions moved.

skin, the latter being the structure responsible for the transcapsular potential and active transport. If this were true and the principle were of general importance, then in specialized systems having epithelial cells with a basement membrane (for example, lens, corneal epithelium, frog skin, ciliary processes, kidney tubule cells, and so forth) it is proposed that the potential is across one continuous membrane, the basement membrane. The basement membrane envisioned here would be an active metabolic membrane of high impedance and capable of active transport of ions; it might be originally secreted by and maintained by the epithelial cells throughout life. The purpose of such a system would be to prevent the need for the epithelial cells themselves from having to be permanently asymmetrically polarized.

Meyer and Bernfeld[23] first proposed that the frog skin potential was located across the basement membrane. Ottoson, Sjöstrand, Stenström, and Svaetichin,[24] using the microelectrode technique, concluded that the potential of frog skin was located entirely across the basement membrane; they found that the essential skin potential was obtained in one potential jump near the dermo-epidermal junction. These investigators obtained electron micrographs of frog skin showing a hitherto undiscovered true basement membrane 250 Å thick and about 200 Å from the basal epithelial cells; they further showed that the basal epithelial cells were not a syncytium but had intercellular spaces between them. Linderholm[25] also proposed that the essential diffusion resistance in frog skin is due to the single thin basement membrane. He considered the basement membrane as the site of the Na^+ pump and the skin potential; he further postulated that the chemical reactions involved in the active transport mechanism may be driven by the epithelial cells.

Ussing[26] suggested that the site of active transport occurred at the basal membranes of the basal epithelial cells. Koefoed-Johnsen and Ussing,[27] however, first suggested that the potential of frog skin, bathed in a solution in which Cl^- was replaced by nonpenetrating $SO_4^=$, was the sum of a Na^+ diffusion potential at the outer border and a K^+ diffusion potential at the inner border of the basal epithelial cell. This model involves an asymmetrically polarized epithelial cell, that is, the inner and outer surface membranes are at different potentials relative to the interior of the cell. Engbaek and Hoshiko[28] tested this model by using the microelectrode technique to determine whether there were one or two potential jumps on piercing the frog skin bathed in $SO_4^=$-Ringer. These investigators reported that the total skin potential was attained in two or three potential jumps at different depths in the skin; they presumed the two jumps were situated at the outer and inner borders of the same epithelial cell.

Hence the controversy over frog skin is not settled. However, there are attractive aspects to the basement membrane hypothesis and one might speculate whether such a structure exists at the inner border of the lens capsule. Electron microscopy of the lens capsule being done in conjunction with another laboratory may reveal such a basement membrane. A basement membrane has been found for the corneal epithelium distinct from Bowman's membrane.[29] Pappas and Smelser[30] reported that the outer epithelial layer of ciliary processes has a basement membrane.

There is some histologic support for a basement membrane in lens capsule if special interpretation be given to published photomicrographs. Jess[31] in a study of copper cataract described a green layer about 0.3 μ thick deposited between the capsule and epithelium; since this was the only area heavily stained, it might be indicative of a separate anatomic structure. Lindner and Böke[32] published a phase micrograph showing the relationship between the capsule and epithelium; they neglected to call attention to a heavy dark line between the epithelial cells and the body of the capsule; this dark area may represent the position of a basement

membrane. These investigators and Bahr[33] published electron micrographs of the capsule showing a regular lamellar structure. Wislocki[34] described a pericapsular membrane at the outer border of the capsule.

Summary

1. Bioelectric potentials associated with the lens have been studied with wick electrodes and conventional microelectrodes. Transmembrane potentials of the lens fibers in bovine eyes were found; they had a mean value of -23.3 ± 1.2 mv., negative inside. A transcapsular potential was also found; for bovine lenses in situ this potential had a mean value of -30.2 ± 1.4 mv., negative inside. Another potential averaging about -10.3 mv. was observed between the extracellular space and the bathing medium. The three potentials described were in series, hence the potential between the inside of a fiber and the outside of the capsule is the sum of the three or -63.8 mv.

2. The posterior capsule was found to have a DC resistance of about 1600 ohms/cm.^{2}; the "short-circuit" current was determined to be about 18 μamp./cm.^{2}, theoretically equal to the maximum active transport current.

3. The transcapsular potential and resistance were reduced essentially to zero in local areas by mechanical injury, hyperosmotic solutions of univalent electrolytes, potassium ion (150 mEq/l), and cathodal current; this enabled the transcapsular potential to be recorded externally as an injury potential.

4. With some, but not all, of the localized capsular injuries, localized opacities of the superficial cortical fibers appeared rapidly beneath the injured area. However, capsular injury and immediate opacity have no direct relationship to each other.

5. Temperature had a marked effect on the transcapsular potential, the maximum potential being generated at body temperature and falling off rapidly at lower temperature. Ultraviolet light, 2:4 DNP, IAA, and EDTA decreased the transcapsular potential about 50 percent; uV and EDTA were used to decrease the fiber resting potentials about 40 percent in one hour. It is concluded that the potentials are dependent upon metabolic energy.

6. Replacement of Na^+ by K^+ in the bathing medium abolished the transcapsular potential but produced no clouding; replacement of Na^+ by choline$^+$ had no effect on the potential but produced reversible clouding. Replacement of Cl^- by $SO_4^=$ produced about 50 percent decrease in potential in one hour and no clouding; double replacement of Na^+ and Cl^- by K^+ and $SO_4^=$ produced a slightly positive potential and a marked reversible clouding. From the ion replacement studies it is concluded that the transcapsular potential is mainly an expression of the K^+ equilibrium potential.

7. It is concluded that the lens capsule is not an inert, passive structure but rather is an active membrane of considerable resistance, and somewhat equivalent to a cell membrane. Possibilities for the discovery of a basement membrane at the inner border of the capsule were discussed.

8. It is further concluded that the K^+, Na^+, and Cl^- ions are not uniformly distributed within the lens, but rather electrochemical gradients of these ions exist across the membranes of the lens fibers. Hence the latter probably are sites of active ion transport and possess some electric properties similar to nerve and muscle fibers. In this respect, the organization of the lens is not as one giant cell with the capsule as the cell membrane, but rather of many individual cells within one giant cell.

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IN VITRO LENS STUDIES: III. LENS POTENTIAL*

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In a previous study[1] certain dyes were observed to produce subepithelial vacuolar changes and/or cytopathologic changes in the epithelium of rabbit lenses cultured in vitro. The basic dyes (methylene blue, thionin, toluidine blue) were cataractogenic, whereas the acidic dyes (indigo di- and tetrasulfonates) studied were nontoxic. An exception was 2,6 dichlorophenolindophenol, an acidic dye, which caused marked cloudiness and vacuolar changes. The cataractogenic dyes had a common property, namely a positive oxidation-reduction potential when 50-percent reduced (E_o). Therefore, it was of interest to determine whether these agents affected the oxidation-reduction potential of cultured lenses. In addition, measurements of the glutathione concentration of lenses were made in order to ascertain whether further similarities were evident between cataracts produced in vitro[1] and in vivo.[2,3]

Methods

Lenses were cultured as previously described.[4] The culture procedure consisted essentially of incubating the whole lens in TC199 media (Difco) containing approximately 20-percent supplemental buffer (tris-hydroxy-methylaminomethane, pH 7.5). The potential was measured with a platinum electrode. The electrode was constructed by connecting 32-gauge platinum wire to the platinum wire lead of the Beckman glass electrode. It was made rigid to within 1/16 inch of its tip by a glass capillary and insulated to approximately 0.5 mm. or less of the tip with a thin layer of DeKotinsky cement. Potentials of reference hydroquinone solutions by this electrode were within 5.0 mV. of those obtained with standard platinum electrodes. A Model GS Beckman pH meter was used. In order to penetrate the lens capsule it was necessary to sharpen the tip and to apply gentle pressure against the lens with a polyethylene-coated loop as the lens was carefully but sharply jabbed with the electrode tip. The potentials were taken at slightly less than 1/16 inch depth into the cortex. The lens was immersed in media at room temperature. Potentials reported are the average of two and generally three determinations of each lens at different foci in each area, peripheral (one to three mm. from equator) or central as noted, and refer to the saturated calomel half-cell in the media. Glutathione was measured by the nitroprusside method of Grunert and Phillips,[5,6] using precipitating solutions saturated with nitrogen.

Results

Measurements of the potentials of lenses cultured in basal media and in the presence of various dyes for 12 hours are summarized in Table 1. The average potential at the peripheral one to three mm. of lenses cultured in basal media was found to be -78 mV. The central area was in general found to be slightly more negative. Potential measurements at the peripheral or central areas of the majority of control lenses did not differ by more than 10 mV. in a series of three to four measurements at each area of an individual lens.

Cationic dyes caused the lens potential to become more negative by some 100 mV. in the peripheral portion of the lens. The central area was less affected and was an average of approximately 30 mV. more negative than control lenses. No difference was apparent in these series of experiments be-

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TABLE 1

POTENTIAL OF LENSES CULTURED FOR 12 HOURS IN THE PRESENCE OF VARIOUS "CATARACTOGENIC" COMPOUNDS

Compounds Added to Media[1]	Number of Lenses	Lens Potential[2] Peripheral	Lens Potential[2] Central
		mV Ave ± S.D.	mV Ave ± S.D.
Controls—media only[3]	13	− 78 ± 27	− 88 ± 38
Cationic dyes			
Methylene blue	4	−172 ± 29	−121 ± 38
Thionin	4		
Toluidine blue	4		
Anionic dyes			
2,6 dichlorophenolindophenol	4	− 46 ± 21	− 49 ± 21
Indigo di- and tetrasulfonate	4	− 70 ± 16	− 81 ± 27
Triphenyltetrazolium	3	− 65 ± 25	− 70 ± 20

[1] Concentration of 2×10^{-5} M, except triphenyltetrazolium, 1×10^{-4} M.
[2] With reference to standard calomel electrode.
[3] The controls were cultured contralateral lenses. The omissions were primarily from the cationic dye series with one from the anionic dye experiments and two from the triphenyltetrazolium series.

tween the effect of methylene blue, thionin, or toluidine blue.

Anionic dyes caused negligible changes in lens potential. These lenses also showed a definite tendency for the central area to be more negative than the periphery as found in control lenses in contrast to the effect of cationic dyes. The apparent decreased potential in the 2,6 dichlorophenolindophenol series was due to the fact that the lenses of the majority of the rabbits used in these particular cultures had less negative potentials than was generally found. The contralateral lenses in basal media showed similar potentials. Repeated experiments (table 2) showed no difference between the potentials of lenses cultured in the presence of 2,6 dichlorophenolindophenol and the contralateral lenses cultured in the basal media.

The potentials of lenses cultured in media containing anionic dyes (indigo) which were non-toxic to lenses were in good agreement with those of control lenses cultured for a similar period. These results indicated a stability of lens potential during a 12-hour culture period. This point was further checked by measuring the potentials of lenses cultured for 12 hours and of the contralateral lenses immediately after removal from the eye. These data (table 2) indicated that the culture procedure had no appreciable effect on the lens potential. However, in one series of lens cultures the potentials of the cultured lenses were slightly more negative (an average of −12 mV.) and in a second series slightly less negative (average of +11 mV.). Thus, it appears that further study along this approach is necessary in order to add this criterion for successful lens cultures to those already established.[4]

During earlier studies on the production of vacuolar changes it was observed that the uptake of dye by the epithelial cells was not proportional to their cataractogenic effect. Severe changes were frequently seen at four hours and the more intense uptake of dye during longer cultures masked their presence.[1] It was also observed that the very small amount of dye carried on the loop after inserting the lens into the media was sufficient in many instances to be toxic. Consequently, thorough precautions in the washing of glassware and instruments were necessary to assure the successful culture of control lenses without vacuolar changes occurring. In the present study the effect of different amounts of a cationic dye (methylene blue) on the potential of lenses cultured for

TABLE 2

POTENTIAL AND GLUTATHIONE CONTENT OF LENSES CULTURED FOR VARIOUS TIME INTERVALS

Compound Added to Media	No. of Lenses	Culture Period	Lens Potential (Periphery)	Glutathione Content	Lens[3] Wt.
		Hours	mV Ave ± S.D.	mg./100 gm. Ave ± S.D.	mg. Ave.
Uncultured	8	0	− 73 ± 13	362 ± 30	441
Media only	8	12	− 74 ± 15	353 ± 33	435
Media only	8[2]	4–8	− 57 ± 18	367	(400)
+methylene blue (trace[1])	8[2]	4–8	− 86 ± 10	337	(400)
Media only	4	8	− 53 ± 14	461 ± 15	383
+methylene blue (10^{-5} M)	4	8	−163 ± 40	324 ± 68	383
Media only	4	12	− 75 ± 3	453 ± 85	365
+methylene blue (10^{-5} M)	4	12	−209 ± 9	234 ± 42	374
Media only	4	12	− 83 ± 16	376 ± 20	491
+2,6 dichlorophenolindophenol (10^{-5} M)	4	12	− 88 ± 12	371 ± 10	489

[1] Concentration was only a fraction of that used in other experiments. Most lenses were unstained and others showed very faint punctate staining of cells (53× magnification examination) in contrast to the moderate to marked degree of staining at 10^{-5} M. These unstained lenses, however, showed marked degree of vacuole formation.

[2] There were 4 pairs of lenses at each time interval used for potential measurements. The glutathione measurements were made on 2 pairs of these lenses at 4 and 8 hours each and 6 pairs of lenses from other 4-hour cultures. An average lens weight of 400 mg. was used for calculations.

[3] With the exception of the uncultured lens, weights were obtained after the lens was cultured in order to avoid contamination and undue delay in getting the lens into the culture media after enucleation. A Roller-Smith micro torsion balance was used.

four, eight, or 12 hours was studied (table 2). The amount of methylene blue used in trace quantities was insufficient to cause any visible (×53 magnification) staining in most instances and only very fine stippling in others. The potentials of the experimental lenses were more negative by an average of 29 mV. than those of the contralateral lenses cultured in basal media. This alteration in potential was considerably greater than that found in control experiments of uncultured versus cultured lenses (average of −1.0 mV.) or in the experiments using 2,6 dichlorophenolindophenol (−5.0 mV.). The trace quantity of methylene blue caused moderate to severe vacuolar changes. However, in individual lenses the potential change did not appear to be directly correlated with the severity of the vacuoles, although the latter criterion is difficult to evaluate quantitatively. It was more evidently correlated with the presence of staining which occurred principally at the periphery of the lens.

This relationship to staining was demonstrated by the cultures with 10^{-5} M. concentration of methylene blue. The potentials of these lenses were more negative than those of the contralateral lens by approximately 110 to 134 mV. on the average. Although the average potential change was greater in the 12-hour cultures, occasional lenses cultured for eight hours showed a similar very negative potential.

Because of the generally reported decrease in glutathione content of lenses preceding or concurrent with cataract development in vivo, it was of interest to determine whether the concentration of this important metabolite was associated with cataractous changes produced in vitro. Data pertaining to the glutathione concentration of cultured lenses are summarized in Table 2. In comparing the glutathione content of uncultured and cultured lenses, it appeared that the glutathione content of lenses was not significantly altered during the 12-hour culture period.

Culturing lenses in media containing traces

of methylene blue resulted in a more negative potential and a decreased glutathione content. Although these changes occurred in each instance, the lens having the greatest change in lens potential did not in each such instance have the greatest loss of glutathione. However, lenses cultured in media containing 10^{-5} M. methylene blue showed a marked drop in lens potential and a greater loss of glutathione (30 percent) in eight hours which progressed to an average loss of 48 percent at 12 hours. Although the methylene blue concentration in the media was not measured after culture, it was by no means completely reduced. Visual estimation of the amount reduced might approximate 10 to 20 percent of the dye although a portion of this loss was represented by staining of the epithelium. On the other hand, the presence of 2,6 dichlorophenolindophenol which has an E_0 of +0.175 to +0.203 at the pH's possibly encountered during culture had no significant effect on the lens potential or the glutathione content of lenses in these experiments. This dye was generally almost completely reduced by 12 hours with no staining of the lens.

Lenses cultured for 12 hours in the basal media showed an average decrease in weight of 1.3 percent from that of the contralateral, uncultured lenses. Methylene blue at 10^{-5} M. concentration caused a slight increase in lens weight (less than one percent) of three of four lenses at eight hours when compared to the contralateral lenses cultured in basal media. At 12 hours a somewhat increased weight (2.5 percent) was observed. The effect of 2,6 dichlorophenolindophenol was variable. Two lenses showed a decreased weight (2.0 percent)and two showed an increased weight (1.4 percent).

Discussion

In establishing a successful lens culture technique, it was felt that as many criteria as possible and applicable should be used to prove the viability of the cultured lens to be identical to its status in situ. Previous studies[1,4] employed the criteria of mitotic activity, absence of vacuolar changes, glycolytic activity, and clarity. Those experiments indicated that TC199 media containing supplemental buffer maintained the lens in culture for 12 to 16 hours in a state almost identical to that in vivo. The more sensitive criteria were mitotic activity and freedom from vacuolar formation. Although mitotic activity was routinely successfully maintained, it has not been possible to culture lenses free from subepithelial vacuoles as a routine procedure. The present studies indicated that on the basis of two additional criteria, lens potential and glutathione concentration, the rabbit lens may be maintained in vitro for 12 hours in a similar status as in vivo.

The principal factors maintaining the lens potential are not clearly established. The reduced metabolites, glutathione and ascorbic acid, have been assigned this function by some investigators. Rosner, et al.,[11] found that in synthetic mixtures of glutathione and beta-crystallin the lens potential became less negative as the glutathione concentration decreased. Ascorbic acid did not influence the potential of the mixtures. On the other hand, Nordmann[8] noted that the lens potential became less negative in scurvy correlating with the disappearance of ascorbic acid. Following a period of instability, the lens potential may then become more negative than normal despite the continuing absence of ascorbic acid. Galactose cataract in the rat[3] was associated with a less negative lens potential as was naphthalene cataract or dinitrophenol toxicity without opacities in the rabbit.[9] Glutathione was found to be reduced in galactose cataracts[3] and x-irradiation.[2] The loss of glutathione preceded the clinical appearance of cataracts.

In the present in vitro studies, cationic dyes caused a considerably increased negative potential and a representative of this group of dyes, methylene blue, caused a significant loss of glutathione. These effects apparently were not due to the positive E_0 potentials of these dyes per se (methylene blue +0.01, thionin +0.06, toluidine blue +0.01)[12] as

2,6 dichlorophenolindophenol with an E_0 of $+0.217$[12] was ineffective. The loss of glutathione may be related to the possible very slight penetration of the lens by methylene blue. However, the loss of this reduced metabolite would persumably result in a less negative potential rather than the increased negative potential found.

An effect of dyes on ion transport in other systems has been observed. Cationic dyes have been found to decrease the uptake of sodium by the isolated gills of Eriocheir sinensis.[13] Methylene blue has also been observed by Taylor, et al.[14] to increase the leakage of potassium from erythrocytes without affecting the rate of uptake. These effects were presumably associated with the inhibition of cholinesterase by methylene blue. The penetration of isotopic sodium into the lens has been interpreted by Brindley[7] as a continual leaking of sodium by the lens and its active transport against a concentration and potential gradient. Cataractous lenses and death of the lens is well known to be associated with the loss of potassium and the accumulation of sodium. Similar reversal of the normal state was also associated with cooling or metabolic poisons.[15] These cation exchanges would in part account for the less negative lens potential found in in vivo cataracts. Cationic dyes possess other toxic properties among which is the inhibition of dehydrogenases[16,17] and the prevention of formation of high energy phosphate bonds.[18] Without additional information on the cation content, the protein-SH, ascorbic acid concentrations, and so forth, interpretation of the effect of the "cataractogenic" compounds used in these studies on cultured lenses can only be speculative.

On the basis of mitotic activity inhibition, cytopathologic changes (methylene blue and triphenyltetrazolium), vacuolar formation, and decreased glutathione concentration, it would appear that the cataractous changes produced in vitro may bear some resemblance to those produced in vivo. However, the marked increase in negative potential of the lens in vitro indicates a dissimilarity from in vivo cataracts unless a potential reversal occurred as described by Nordmann in scorbutic animals. In addition, it is apparent that vacuolar formation may occur without affecting any of the five criteria used in these studies. These vacuoles which occur initially at the extreme periphery of the lens in the germinative zone are readily seen in the isolated lens under high magnification but may well be hidden behind the iris when the pupil is maximally dilated.

Summary

The effect of cationic and anionic dyes on the potential and on the glutathione content of cultured lenses of rabbits was studied.

The potential and glutathione concentration of cultured lenses was in very good agreement with that of the contralateral lens immediately after enucleation.

Cationic dyes had a marked effect on the potential of the lens. The potential became more negative by approximately -120 mV. Lenses cultured in media containing 10^{-5} M. of methylene blue also showed a considerable loss of glutathione content. The decrease in glutathione concentration averaged 48 percent at 12 hours.

Anionic dyes showed no significant effect on the lens potential in these experiments. The glutathione content of lenses cultured in media containing 10^{-5} M. of 2,6 dichlorophenolindophenol showed no significant difference from control lenses.

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Discussion*

Dr. Bernard Becker (St. Louis): I find these three papers most exciting, and I am a little at a loss to explain or attempt to correlate the results obtained.

It is understandable that an agent such as methylene blue, that induces early cataractous lens changes, interferes with cation transport, decreases glutathione, and leads to vacuolization, cytopathology, and altered miotic activity. One would expect such an agent to reduce the negative lens potential. However the increase in the negativity of this potential noted by Dr. Constant is difficult to explain on the basis of ion transport.

Dr. Harris pointed out that there is an increase in glucose utilization following methylene blue, and it is tempting to speculate as to the relationship of this to the increased negative potential. By analogy with the red cell, there is the possibility that this increased glucose utilization may take place via the shunt. I would like to ask Dr. Harris whether there is evidence in the lens for an increase in shunt activity in the presence of methylene blue. Is there also evidence in the lens for an increased oxygen consumption, as there is in the red cell in the presence of methylene blue? However I am a little concerned about the analogy with the red cell. As I understand, Maizels (J. Physiol., **112**:59, 1951) found that methylene blue does not alter sodium transport in the human red cell, even when oxygen and glucose consumption are increased.

The exciting thesis Dr. Harris proposes, of a possible photodynamic action of methylene blue, also raises many questions. First, one would like to know what darkness does to some of the other findings that follow methylene blue. Do we see the cytopathology, the mitotic changes, the reduction in glutathione, and the increase in negative potential if the methylene blue is applied to the lens in the dark? I am sure these are experiments that pass through the minds of most of you and are certainly contemplated.

There is another question I would like Dr. Harris to answer. There is some literature on the effects of methylene blue on synthesis of acetylcholine, rather than on its degradation by cholinestrase. This has been postulated as a possible mechanism for altering the cell surface of red cells. Is there evidence (and I express ignorance in this matter) for a choline acetylase in the lens? Is this altered by methylene blue?

Finally, an increase in negative potential in the face of alterations in metabolism suggests the possibility of some uncoupling mechanism. In the ciliary epithelium, for example, when the animal is subjected to adrenalectomy or ascorbate deficiency, the difference in potential between the stroma and epithelium increases. This is postulated to be a lack of interaction between stroma and epithelium. Does methylene blue act as an uncoupler?

Dr. D. Venkat Reddy (Detroit): I would like to confine my remarks primarily to the paper by Dr. Sperelakis and Dr. Potts.

We, too, have made some observations on the rabbit lens potential that are in essential agreement with the authors' work and also that of Brindley.

* This discussion includes the preceding three papers.

Although we have not measured the potentials of the fibers and the lens capsule separately, and our measurements record the combined potentials, we find that by simple irrigation of the lens with one molar KCl we cannot only abolish the negative potential but also convert it to positive polarity. I was curious to note from your data that only a third of the potential was abolished across the membrane of the fiber when the lens, decapsulated or otherwise, was incubated in KCl solution.

Dr. Harris has mentioned that increased glucose utilization resulted in an increase in the negative potential of the lens capsule. All available information on the metabolism of the diabetic rabbit lens points to the fact that there is an impairment of glucose metabolism. I wonder whether Dr. Sperelakis and Dr. Potts have made any studies on diabetic lenses to see whether there is a decrease in the transcapsular potential.

The authors should be congratulated for their success in demonstrating electrochemical gradients within the lens and the measurement of short circuit current across the capsule. It is unfortunate that they did not succeed in carrying out this study further by actual measurements on the isolated capsule. They may take comfort from the fact that our own efforts in this regard have failed repeatedly.

Dr. Jin Kinoshita (Boston): I am supposed to discuss Dr. Constant's paper but, with your permission, I would like to discuss these three papers together because I agree with Dr. Becker that they touch on a phase of study which may prove to be one of the most significant areas of experimentation in lens physiology.

I think the common property touched on by these papers is the permeability barrier of the lens. Apparently this permeability barrier requires metabolism to maintain its viability, and without the necessary metabolic processes the barrier breaks down.

Now let us consider Dr. Harris's situation, in which he uses refrigerated lens. I have often wondered how the metabolism of a lens refrigerated for 24 to 48 hours compares with the normal lens. Some preliminary studies I have made indicate that storage of lens in the cold for 24 hours greatly reduces the metabolic activity measured at 37°C. If this is the case is it possible that the metabolism of the lens in general is not associated with the shift of cations to the normal state by these lenses when placed in a more normal physiologic environment? Perhaps the lens barrier has its own energetic mechanims which are not related to the general lens metabolism.

The other thing I would like to mention about these three papers is the lens potential. I think the other work which would have to be considered along with these papers is the significant work of Dr. Brindley. He suggests that, rather than an oxidation reduction potential, the resting potential observed in lens is an electrochemical one generated by differences in concentration of cations and anions inside the lens, as opposed to those in the environmental fluids. I think this is in quite good agreement with the Cleveland group.

In view of this, I am rather confused as to Dr. Constant's indirect implication, at least, of trying to tie up the relationship of glutathione with the lens potential, and I wonder if glutathione actually contributes very much to the resting potential of the lens.

I agree with Dr. Sperelakis, in view of Brindley's work, that the potassium battery is the most essential contributing factor to the lens potential.

The third aspect is the consideration of the effect of methylene blue and the effects which were observed by Dr. Harris and Dr. Constant. From the presentation of these data by Dr. Harris, in which he shows that other cationic dyes with positive oxidation reduction potentials show the same effect as methylene blue suggests to him that the property involved is the oxidation reduction potential of these dyes. I wonder if it is of any significance that these dyes with such different E_o have the same effect as methylene blue. If this effect involves an oxidation reduction potential I would have thought it would require a greater specificity. Perhaps the cationic property or a photochemical product is more directly involved than the oxidation reduction property of these dyes.

Dr. Herbert Kaufman (Bethesda). These experiments are extremely interesting, and I admire Dr. Harris's insight in realizing the importance of light.

The subject of photodynamic inactivation is an old subject. In 1898, Raab first observed it in relation to paramecia exposed to the methylene blue, and since that time a large body of knowledge has accumulated on the subject. Dr. Aronson, at the National Institutes of Health, has found that when tissue cultures are exposed to light in the presence of acridine orange dye they will be killed, whereas cultures not exposed to light live on.

In understanding the dynamics of the phenomena observed by Dr. Harris, and perhaps also by Dr. Constant, some knowledge of the nature of the photodynamic effect would be desirable. Dr. Harris postulates that one of the effects may be a direct damage to protein membrane. Mrs. Kaufman, working with Dr. Hiatt and Dr. Helprin at the Natonal Institutes of Health, has been experimenting with photodynamic inactivation of bacteriophage.

When exposed to light for just a few minutes, in the presence of toluidine blue, the phage appears inactivated. Electron micrographs suggests that the light has caused a large defect in the membrane of the head of the phage (fig. 1).

Since this happens so rapidly, it would certainly support the theory reported by Dr. Harris that destruction of a protein membrane may be a primary occurrence. The rapid occurrence of this change in phage, and the failure of toluidine blue impregnated controls unexposed to light to exhibit such a change, suggest that this is not secondary to enzymatic defects produced by the dye.

It seems possible also that this photodynamic effect may have some bearing on Dr. Constant's observations.

Dr. Gerhard A. Brecher (Atlanta): I want to ask Dr. Harris if he has made any specific study

concerning the spectral quality of the light which activates the methylene blue.

For example, which energy levels were used? Also, was ultraviolet light used or excluded? Which specific spectral quality of light is the one that does the activation? Also, the time course of this would be of interest in further study.

DR. JOHN E. HARRIS (Portland, Oregon): To answer the last question first, there is, of course, a direct relationship of the action spectrum of the light to the absorption of the methylene blue. We have used just visible light, not ultraviolet light. Actually, we haven't been too fussy about our light source since this is not necessary. We have simply used ambient light.

We have made no measurements of the number of quanta required to produce the measured effect. These figures would be interesting. The quantitative aspects of photodynamic hemolysis have been worked out to a fair degree of certainty. The exact data I am afraid I cannot quote at the moment. However, the number of quanta required to lyse one red cell is quite appreciable.

While thinking of it, Dr. Becker, methylene blue affects the cation balance of the erythrocyte, as shown by some workers in Hastings' laboratory. However, they did not check the photodynamic activity. This was done with rose bengal years ago, as I mentioned.

As Dr. Becker has mentioned, methylene blue inhibits both choline-acetylase and cholinesterase. I know of no data that would indicate that this is of any significance in the lens.

I am intrigued by the potential measurements of both Dr. Constant and Dr. Sperelakis. I would quite agree with the conclusion that there is a transport across both the fiber and the capsule as well. The transport across the capsule is probably essential if we are to maintain normal hydration, otherwise I can't quite conceive of how we are going to control the hydration between the fibers themselves. When cation transport is blocked at the lens surface we do find sometimes an accumulation of fluid underneath the capsule and between fibers. Most of our data are compatible with a transport at the lens surface only and the data of Dr. Sperelakis and Dr. Potts, which indicate a potential across the fiber membrane, are really interesting. Hydration is determined by cation transport and both intra- and extrafiber hydration imust be controlled if lens clarity is to be maintained.

I don't think we can explain right now the effect of the methylene blue on the potential Dr. Constant has measured. I would not, however, rule out the possibility that this is not some cationic effect. If you will recall the equation that Dr. Sperelakis mentioned, there is a factor here of the diffusion constant, or the potassium flux.

In our experiments just the total amount is measured. We cannot determine the exchange rate from our studies. This can be shown only by using radioisotopes. It is within the realm of possibility that under the conditions which Dr. Constant employed an increase in exchange may have occurred. If you will recall, in some of our studies where we cultured them, as Dr. Constant has done, the data were variable and many showed little change in potassium concentration. Further studies will be necessary to determine that the potential she has measured is due to the potassium battery.

Fig. 1

DR. NICK SPERELAKIS (closing): Perhaps the major discussion here should be a clarification of the distinction between a redox potential and a non-redox electrolytic potential. The redox electrode system (calomel half-cell and platinum) used by Dr. Constant is entirely different from that used by us (two Ag:AgCl half-cells). Potential measurements with redox electrodes do not indicate ion distributions, but rather depend upon the logarithm of the ratio of oxidized to reduced substances; hence redox compounds like methylene blue added to the outside medium will have an effect upon the redox potential because methylene blue is positively charged. There is little analogy between redox potentials and non-redox electrolytic potentials.

Of course we are well aware of Brindley's work (1956). We used an electrode system similar to his. We have subdivided the 65 mv. "resting" potential he obtained into two separate potentials, namely, the transcapsular and the transfiber potentials, the sum of which approach his value. Brindley probably has recorded both potentials simultaneously. It should be noted that he reported no results using decapsulated lenses. Dr. Constant's electrode system, I believe, will measure the sum of the redox potential and the non-redox transcapsular potential. It is certainly very difficult to make any analogies between potential measurements in her system compared to Brindley's or ours.

In answer to Dr. Reddy's question as to whether we had attempted to record transcapsular potentials from isolated anterior capsules, we did try this.

Such a preparation would be much better than frog skin, in fact it would be a nearly perfect system in which to sutdy active ion transport and the origin of bioelectric potentials. However, I regret that, out of many attempts, only one experiment appeared to be successful although even it may have been an artefact. In this one experiment we were able to obtain a rise in transcapsular potential from an isolated anterior capsule mounted in a Ussing-type holder by an increase of temperature; this potential rapidly declined upon cooling. From the results described in our paper concerning capsular injury and resistance, it can be assumed that excision of the capsule injures it markedly.

The work of Dische (1955) has shown that the ATPase activity in the lens capsule increases markedly when the capsule is isolated, hence some biochemical changes are occurring. Furthermore, the report of Harris (1954) also has shown the phenomenon of caspular injury. He reported that a "nick" to the lens caspule produced a rapid cation shift. Again this is marked evidence that the capsule injures very easily, and the injured area acts as a short-circuit to the potential due to the capsular resistance decreasing to essentially zero.

This is somewhat analogous to the "injury" potential of nerve or muscle: if one end of a frog sartorius is crushed or placed into isosmotic KCl, an "injury" potential is obtained between the normal area and the injured area. The fact that a lens "injury" potential can be otbained by merely injuring a local area of the capsule with a piece of filter paper soaked in hyperosmotic saline is consistent with the view that there is truly a potential across the capsule, the latter not being an "injury" potential recorded from lens fibers.

In regard to Dr. Reddy's other questions, we did not study diabetic lenses. However, we did several experiments on x-radiation of rabbits in order to produce pre-cataractous lenses. The results indicated that up to four days after whole-head irradiation with 1,000 r no effect of radiation damage was reflected in the transcapsular potential. We did not pursue this study further. The effect of isosmotic KCl on the transcapsular potential is very rapid, whereas, in the case of the fiber potentials, the effect is only partial in short times perhaps due to the time needed for the KCl concentration outside the fiber being measured to equilibrate with the KCl in the bathing medium.

In answer to Dr. Harris's question, I don't think that species differences account for differences between Dr. Constant's results and ours. We have studied several species, including rabbit, frog, and bovine lenses. We also measured several rabbits under nembutal anesthesia and found that the transcapsular potential is roughly similar to what one obtains from rabbit or bovine eyes several hours post-removal.

I might add that we have made a rough calculation from Dr. Harris's curves (1954) of lenticular cation shifts. After refrigerating rabbit lenses about 24 hours, he found a cation shift, and upon bringing the lenses back to body temperature the cation shift reversed. Following that reversal curve of cation shifts for about three hours, we calculated the rate of active transport occurring under these conditions, which theoretically should be close to the maximum operation of the sodium pump. The calculation indicated that about 270×10^{-11} equivalents of sodium pumped per second across the capsule. This compared reasonably close to our short-circuit current of 18 μamperes/cm.^{2} Assuming sodium to be the cation pumped and assuming the surface area of the average bovine lens to be 6.4 cm.^{2}, our short-circuited current would be equal to 115×10^{-11} equivalents of sodium pumped across the capsule per second.

The results of Frohman and Kinsey (1952), who have shown ATP to be in much higher concentration in the capsule than in the cortical fibers, also indicate that the caspule could be the site of some active process such as active ion transport. Electron microscopy of the lens, being done in collaboration with Dr. Matthews and Dr. Dorman of Baylor University, indicates a highly osmiophilic membrane at the inner border of the capsule as predicted from our electrophysiologic study. Again we hope this will provide additional evidence in favor of a capsular membrane, equivalent to the basement membrane of frog skin (Ottoson, et al., 1953), which may be the site of active transport and the potential difference, rather than the epithelial cells themselves.

Dr. Marguerite A. Constant (closing): May I thank the various discussers for their comments, and answer them in reverse order.

Dr. Sperelakis pointed out the differences between the two types of potential measurements. I was sort of on the spot to prove that the marked negative potential change was not an artefact. If one homogenizes lenses and measures the potential immediately after methylene blue and after incubation, one finds that, contrary to the intact lens, the potential becomes less negative.

In answer to Dr. Kinoshita, the tie-up of glutathione is more incidental than real. We were interested in how much analogy there was to the cataractous lens produced in vitro to that in vivo. It was evident from the increased negativity of the potential that glutathione was not important.

I should like to mention an observation rather than ask a question of Dr. Harris since time is running short. The relationship of staining and cell surface activity of methylene blue was an evident one. Dr. Quastel showed many years ago that if he increased the phosphate content of the media he could block partially the effect of methylene blue. We did this, and there was no uptake of dye by the epithelial cells, but the loss of glutathione and the lens potential change were still evident.

I must confess that his very intriguing photodynamic findings prompted me to take a quick look and see if this affected the lens potential. Apparently incubating the lens in the dark decreases the effect of methylene blue somewhat, but the change in lens potential is still there.

CORNEAL WATER TRANSPORT AS MEASURED WITH TRITIATED WATER*

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The connection between relative corneal dehydration and corneal transparency has been studied by numerous workers since the publications of Leber.[1] In recent years an active transport mechanism has been postulated as the means by which relative dehydration is maintained.[2-5] Using radioactive sodium in the living rabbit we demonstrated a small differential movement of Na^+ from cornea to aqueous[4] but the lack of change on endothelial damage[6] and the absence of a measurable potential difference across endothelium[7] throws some doubt on the interpretation of these results to mean that Na^+ is actively transported across endothelium.

To investigate the alternative possibility that water is actively transported by the cornea, we have been engaged for some time in tracing water movement by the most sensitive method at our disposal, namely the use of a tritium label. Previous work on corneal hydration, of which there is an abundance,[3,8,9] has with one exception been done gravimetrically, and hence has not been precise enough to determine differential flow of water. However, gravimetric methods have been adequate to demonstrate water imbibition at low temperatures and re-dehydration when the same eyes are restored to body temperature—presumptive evidence for active transport at an unindentified site.[3,5] Similar imbibition under the influence of chemical inhibitors of metabolism adds weight to this idea.[3,10,11]

The one attempt at a more sensitive water determination was that of Cogan and Kinsey[12] who employed a deuterium label and who could detect one part D_2O in 2,000 parts of H_2O. This = 1.0/µg. if starting material was 50-percent D_2O. In addition to enhanced sensitivity the use of a label allows measurement of diffusion across a membrane in each direction separately. Cogan and Kinsey reported on the diffusion of D_2O through the cat cornea and gave a figure of approximately 4.0 mm.^{3}/min./cm.^{2} for diffusion from endothelial to epithelial side which was relatively constant over 10 hours and which increased by about 20 percent on removal of epithelium. However, these experiments were done on corneas tied on the flanged ends of pipettes. This procedure caused an indeterminate amount of damage to endothelium, at least, and made evaluation of the roles of the limiting membranes a difficult one.

To allow detection of smaller amounts of water and to follow small volumes of water injected intralamellarly we have been forced to turn to a radioactive label, and we have used tritium labeled water for the purpose. In vivo and in vitro experiments were performed in attempts to evaluate the role of each of the limiting membranes and the stroma. In vitro experiments were done at 0°C. and 27°C. to evaluate the effect of metabolic activity on any possible active transport mechanism. The results of intracorneal injection experiments strongly suggest active transport through the epithelium. A series of further experiments now projected may substantiate this tentative conclusion.

Methods

A. Tritiated water (THO)

Water used in these experiments was prepared in our laboratory from tritium gas by one of two following methods:

* From the Laboratory for Research in Ophthalmology, Western Reserve University, and Eye Service, Department of Surgery, University Hospitals of Cleveland. Supported by a grant (B-30) from the National Institute of Neurological Diseases and Blindness, U. S. Public Health Service. A preliminary report on some of this material was presented before the East Central Section, Association for Research in Ophthalmology, January, 1954.

1. A sealed vial of 50 mc. of tritium gas was sealed in its turn to a system containing two connecting chambers and a side-arm for evacuation. The first chamber contained 10 cc. of water and 50 mg. of platinum oxide catalyst;[13] the second chamber was empty. The system was evacuated to approximately 20 mm. Hg and sealed shut. The break-off seal to the tritium vial was then broken with steel balls previously enclosed and the tritium was allowed to equilibrate with the water for a week. At the end of this time the water was distilled from the first to the second chamber which was then removed from the system as a sealed vial containing highly active THO. There were 10^5 counts/mm.[3] when counted by the method described below.

2. Although extremely simple, an equilibration procedure such as method (1) cannot be a highly efficient way of utilizing the tritium. For this reason we finally turned to a combustion method which gave 100-percent conversion of the radio-activity. Pure hydrogen from a cylinder was burned at a glass jet in an atmosphere of filtered air pulled through a closed tube by an aspirator. To the glass hydrogen tube, just before the jet, had been sealed a vial of 1.0 curie of tritium gas with break-off seal and steel balls. After the hydrogen flame had been ignited by a spark across platinum electrodes at the jet orifice the tritium vial seal was broken and the tritium allowed to mix and burn with the hydogen. The tritium vial was alternately heated and cooled to assure further complete mixing and utilization. The water from the combustion was trapped in a U-tube cooled by dry ice-cellosolve. After 50 cc. of water had been formed the combustion was stopped. The trap was attached to a manifold from which led five vials with glass tubing necks. The water was distilled from the U-trap and partitioned among the five vials. These were then removed from the manifold and, at the same time, sealed with an oxygen torch. Since the cost of commercially tritiated water per millicurie is 500 times the cost of the tritium gas, the preparation is a useful economy.

B. Determination of tritium radioactivity

Tritium has a half-life of 12.5 years, but unfortunately the average energy of the beta rays which it emits is only 13,000 electron volts. This energy is much too small to pass even the thin mica windowed counters used for carbon 14 (whose particle has 10 times this energy). Consequently, some method must be devised to count the radioactivity emitted by the tritium before it can be utilized. No such method applicable to biologic work has appeared in the literature with the exception of a liquid phosphor scintillation procedure requiring expensive electronic equipment. This equipment was not commercially available when our work was begun. Since tritium itself is so inexpensive, and since radioactivity effects of this weak beta need not concern us in an acute experiment of the sort we are conducting, the simplest solution was to use a relatively inefficient method of counting; that is one using infinite thickness of absorber, and to increase the activity of the tritiated water correspondingly.

The method finally devised by us is as follows:

In an ordinary four-inch test tube are placed 1.0 cc. of propylene oxide previously dried over anhydrous nickel sulfate, 50 mg. of anhydrous nickel sulfate ground to an extremely fine powder, and a 10 to 25 cmm. sample of water containing the activity to be determined. The water is dissolved as rapidly as possible in the propylene oxide to avoid evaporation effects. Wherever possible the water is delivered directly into the solvent and mixed well.

The tubes are tightly stoppered and set aside with occasional shaking for a minimum of four hours. Within reasonable limits, there is no maximum time for this step. It is often convenient to do an experiment in the morning, prepare the tubes by afternoon and allow them to stand overnight.

After absorption of the water, the nickel sulfate suspension is transferred by means of a Pasteur pipette to a flat dish of tin plate,

two inches in diameter, and distributed well over the bottom of the dish. The propylene oxide is allowed to evaporate at room temperature and the resultant residue of nickel sulfate containing tritiated water is placed in a windowless gas flow counter.* After the usual flushing with the counter gas, the samples are counted for radioactivity and the results compared with a standard prepared and counted in the same manner.

C. In situ experiments

All experiments were done on albino rabbits under nembutal anesthesia or immediately after being killed with nembutal. The tritiated water adjusted with sodium chloride to the desired tonicity was applied to the eye in one of two ways. It was applied to the anterior surface of the cornea by means of the vacuum applicator previously described,[14] or it was injected intracorneally by means of the microsyringe as also described in our past publications.[4]

When intracorneal injection was used, an inactive solution of 1.35 percent sodium chloride was ordinarily applied to the anterior corneal surface to represent a bath of artificial tears.

At the conclusion of the experiment, the eye was enucleated and frozen immediately. The contents of the applicator were, of course, retained as one of the samples. The eye was dissected while still frozen in a −10°C. room and the component portions of the eye were weighed while still frozen. The liquid components were thawed and the cornea was dehydrated by distilling in vacuo from the cap to the body of a Thunberg tube. The solid sample was placed in the cap, frozen with dry ice, and the tube evacuated. The tube was then sealed and the dry ice bath applied to the tip of the body of the tube allowing distillation to complete itself. The sample thus obtained was absorbed in nickel sulfate as already described.

* Manufactured by Nuclear Measurements Corporation, Indianapolis, Indiana.

D. In vitro experiments

These experiments were carried out on surviving beef corneas excised from the eye with a ring of sclera and clamped by the scleral ring in a lucite holder between two fluid-containing chambers as described previously.[7] With this procedure, neither limiting membrane contacts the holder and trauma is avoided.

The fluid in each chamber was 50 ml. of 0.154 M. $NaHCO_3$ made up with H_2O or THO as indicated. Intralamellar injections were done as in the rabbit except that no sutures were used. Instead the injection was started at a point in the sclera which fell under the clamp, and leakage was prevented by the pressure of the clamp which was applied immediately after injection was complete. At appropriate intervals 1.0 ml. aliquots were withdrawn from the proper chamber (or both chambers after intralamellar injection) and tritium content determined by the method described in Section B above.

Results

A. In situ experiments

The characterisic feature of intralamellar injection into the rabbit cornea was the extreme speed with which water left the cornea (table 1). In five minutes only one percent of the initial activity was detectable in the cornea itself. In the attempt to get some sort of baseline an animal was injected just after being killed with nembutal, the cornea was rinsed, and the eye was enucleated immediately and frozen. Even here, only 20 percent of the initial activity was found in the cornea.

When an applicator was placed on the epithelial surface after intralamellar injection with epithelium intact, the partition favored the cornea-aqueous direction. This differential was abolished when cornea epithelium was denuded. When epithelium was denuded and endothelium scraped with the magnetic flea before injection there was an apparent favoring of the cornea-applicator direction.

Results after applying tritiated water con-

TABLE 1

TRITIATED WATER DISTRIBUTION AFTER INTRACORNEAL INJECTION IN THE RABBIT
Volume = 1.3 mm.³; activity = 9,500 c./m./T.D.

		No. of Eyes	Percent Total Dose			
			Cornea Rinse	Aqueous	Cornea Plug	Applied Solution
A	Dead animal zero time	3	10	65	20	—
B	Dead animal 5 min.	6	12	43	1.1	34
C	Epithelium damaged 5 min.	4	20	30	1.0	30
D	Epithelium and Endothelium damaged 5 min.	4	20	31	1.0	37

tained in the applicator to the epithelial surface are shown in Table 2. Under conditions of these experiments it was not possible to demonstrate a significant difference in the transport of water from tears to aqueous whether or not epithelium was in place.

B. IN VITO EXPERIMENTS

The intralamellar injection into the surviving cornea has the advantage that flow, volume, composition, and temperature of solution can be controlled at both epithelial and endothelial surfaces. The results of intralamellar injection into the beef cornea are shown in Figure 1. The total amount injected was 22.4 mm.³ containing 2.1×10^7 counts/minute. The two pairs of curves show recovery on epithelial and endothelial side when the experiment was conducted at 0°C. and at 27°C. Each point represents no less than two experiments.

It is evident that at 27°C. more than twice as much water leaves the eye via the endothelial than via the epithelial route. Surprisingly, at 0°C. this differential is increased and still more of the injected water leaves via the endothelium, whereas less is found on the epithelial side.

The transport of water through the whole cornea, either intact or with the boundary layers removed, is shown in the remaining figures. Figure 2 shows a control experiment in which sclera was substituted for cornea in

TABLE 2

OCULAR PENETRATION OF TRITIATED WATER FROM ANTERIOR CORNEAL APPLICATOR
Time = 20 min.; area of applicator = 0.79 cm.²; volume of solution = 1.0 ml.

Epithelial Condition	No. of Eyes	Transfer into Aqueous, µg./cm.²/hr.
Intact	6	3.36
Denuded	2	2.96

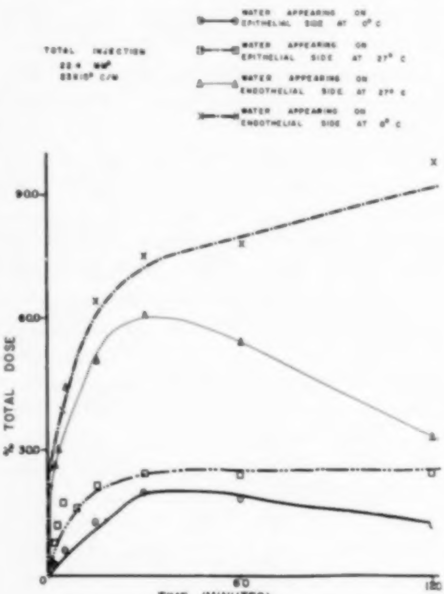

Fig. 1 (Potts, Cohen, and Goodman). Time course of transport of tritiated water after intralamellar injection.

the holder. As can be seen, no activity is detectable for a full 30 minutes after the start of the experiment and by one hour only 40 milli microliters per cm.^{2} have come through as compared to nearly 100 for the cornea. When one considers that the area of sclera exposed in the holder is on the average some two percent of that of the cornea, it may be appreciated that the scleral contribution to water transport in these experiments is truly negligible.

When beef cornea with scleral ring is used in the holder, with labeled water placed in one diffusion chamber and samples removed from the other, one obtains the results shown in Figure 3. It is evident that the order of magnitude of water transfer through the whole beef cornea is much less than that found on intralamellar injection. In the latter experiments we are dealing with rates of 1.9 mm.^{3}/cm.^{2}/hr. for the endothelial direction and 4.9 mm.^{3}/cm.^{2}/hr. for the epithelial direction, as compared to 0.01 mm.^{3}/cm.^{2}/hr. for the through-and-through experiments. It

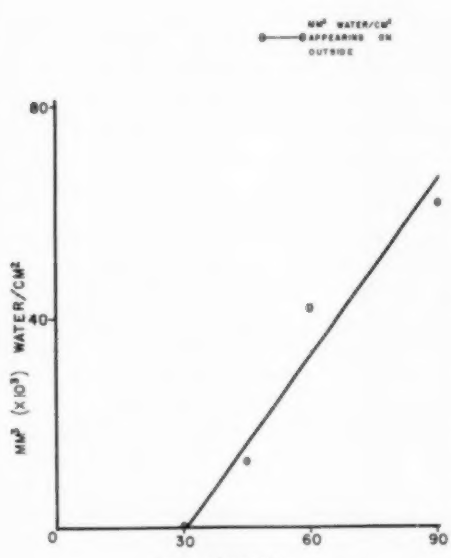

Fig. 2 (Potts, Cohen, and Goodman). Time course of transport of tritiated water through sclera.

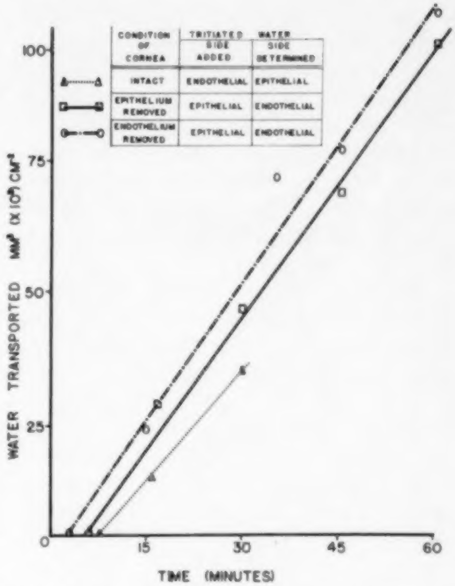

Fig. 3 (Potts, Cohen, and Goodman). Time course of tritiated water through the isolated cornea.

is beyond doubt that this difference in rate is real, for it appears in both the intact rabbit experiments quoted above and the isolated beef cornea experiments.

If one considers the effect of removal of the limiting membranes of the beef cornea, one sees that with membranes intact there is the greatest delay in appearance of the water on the far side and the slope is less than in the membrane damage experiments. With epithelium removed there is increase in slope and less delay in appearance of water. With endothelium removed there is little difference from the epithelium experiment.

Figures 4 and 5 show the effect of ambient temperature on the rate of transport through intact cornea, and cornea with epithelium denuded. In each case the rate of transfer in the cold is less than that at 27°C. The marked delay at 0°C. appears highly significant.

Finally, figure 6 shows that with epithelium removed it makes no significant differ-

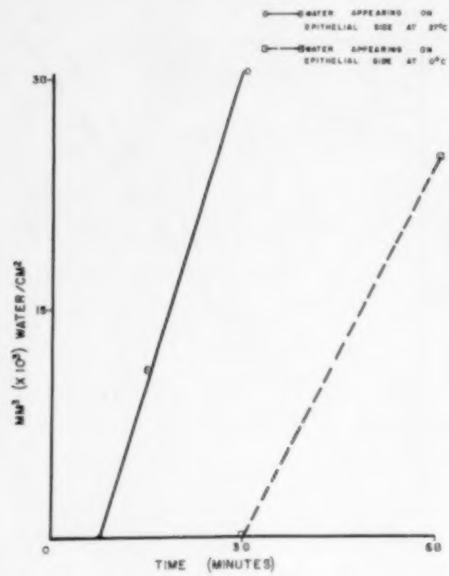

Fig. 4 (Potts, Cohen, and Goodman). Tritiated water transfer temperature effect. I. Intact cornea.

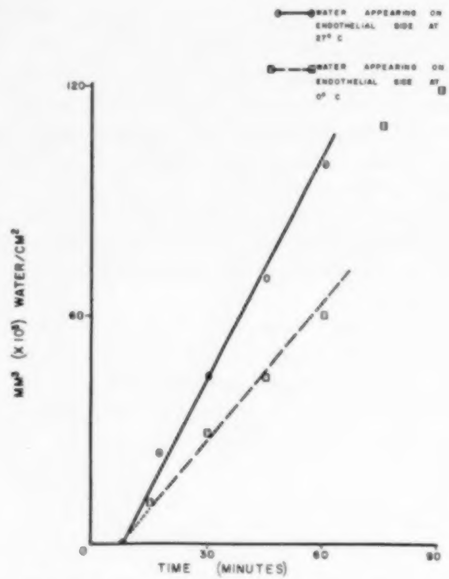

Fig. 5 (Potts, Cohen, and Goodman). Tritiated water transfer temperature effect. II. Epithelium removed.

ence in which direction one measures the rate of fluid transfer; the rate in each direction is essentially the same.

Discussion

One aspect pointed up by the results presented above is the marked difference in the two types of experiment—the intralamellar injection and the through-and-through diffusion experiment. There is a 200 to 500 times difference in rate even assuming that the intralamellar injection utilizes the whole area of the epithelium and endothelium. If less area is involved, the difference is even greater. There must be some additional hydrostatic factor caused by the injection of 22 mm.³ of fluid into the stroma, but this can hardly be the whole story. One important fact is that water injected into the center of the stroma has only half as far to go to either boundary as does water which must traverse the whole thickness. However, there may be an even more fundamental difference in the way a slowly diffusing molecule is handled compared to an injected volume spreading

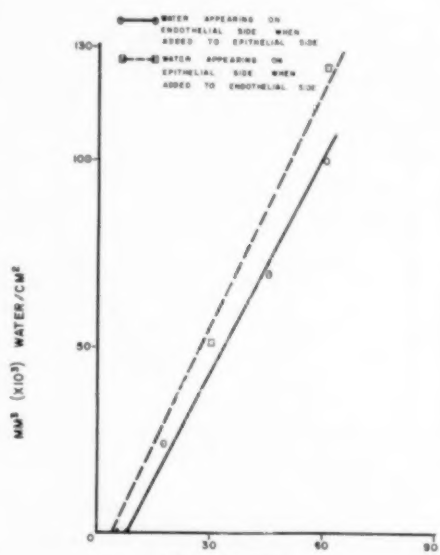

Fig. 6 (Potts, Cohen, and Goodman). Time course of transport of tritiated water through the isolated cornea.

through the extracellular space.

One should also note the marked discrepancy between our whole cornea diffusion experiments and those reported for cat corneas by Cogan and Kinsey. Their figure of 4.0 mm.^{3}/cm.^{2}/min. differs from our beef cornea figures by a factor of 2,400. One wonders whether difference in thickness and density can account for all of this large difference in rate. The increase in transport on removal of epithelium is demonstrated by the experiments from both laboratories.

The temperature effect in the through-and-through experiments is what one might expect if one were dealing with a diffusion constant having a temperature coefficient (Q_{10}) of about 1.2.

The most significant implication of any of the reported experiments is found in the effect of temperature after intralamellar injection. When one works at 0°C. the amount leaving the cornea via endothelium is *greater* and the amount leaving via epithelium is *less* than one finds at 27°C. It is just conceivable that the temperature change has caused a differential change in the passive permeability of the limiting membranes, making epithelium still less permeable than previously and allowing even more water to diffuse out through the endothelium whose permeability has been less affected by the lower temperature.

An alternative explanation is that water was being ejected through the epithelial side by an active mechanism dependent on metabolic energy; that this metabolic activity is inhibited by cold, allowing still more water to escape from the endothelial surface than before. This is with full realization that under the conditions of our experiment epithelial resistance minus epithelial transport is still greater than endothelial resistance to the exit of water.

Experiments are now in progress using other metabolic inhibitors which do not involve temperature change. The results of these experiments should allow a choice between the two alternatives just cited.

A word, too, about the appropriateness of using an isotope such as tritium where there is a relatively large difference between its weight and that of hydrogen. It should be realized that the actual molecule under consideration is THO (molecular weight 20) compared to H_2O (molecular weight 18). Furthermore if we use Thovert's relationship[15] as a first approximation, the diffusion constant varies as the square root of the molecular weight. This would give figures of 4.46 and 4.24 for THO and H_2O respectively. The difference is five percent and should be negligible for purposes of these experiments.

Summary

1. Tritiated water has been synthesized by two different methods and a method for tritium determination has been devised in our laboratory.

2. The transport of water across the cornea and the transport outward after intralamellar injection has been measured in situ in rabbit eyes and in surviving beef corneas held in a plastic holder by a ring of sclera.

3. Water passes from the cornea much more rapidly after intralamellar injection than it does through the cornea by diffusion.

4. After intralamellar injection much more water passes out through the endothelial surface than through the epithelial surface (three to one in isolated cornea). At 0°C. in the isolated cornea this disproportion is even greater.

5. After removing corneal epithelium a greater proportion of water passes out in the epithelial direction.

6. In diffusion experiments with intact cornea, transfer of water is slow and this is even slower at 0°C. The delay in appearance of water is comparable to that seen with sclera at 27°C.

7. Removal of epithelium abolishes this extreme delay and increases rate of diffusion of water through the cornea.

University Hospitals (6).

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Discussion

Dr. Frank Newell (Chicago): The study of Dr. Potts and his co-workers has suggested that corneal dehydration is maintained by an active transport mechanism mediated by the epithelium. The evidence for this theory is the observation that after the injection of tritiated water into the cornea it is injected much more rapidly than when diffusion is the only factor. After interlamellar injection the majority of water passes out through the endothelial surface and at low temperatures an even greater proportion follows this route, and the contribution of the epithelium is markedly decreased. The indication that there is a differential permeability of the corneal epithelium and endothelium, which is eliminated by the removal of epithelium and aggravated by cold, is most suggestive of an active secretory mechanism by the epithelium.

Several questions naturally arise in such a paper. It would be most interesting to learn if the relationship between endothelial and epithelial water transport is linear between 0°C. and 27°C., and also to find the effects of temperatures in excess of 27°C. Secondly, the effect of metabolic poisons on this mechanism which Dr. Potts has mentioned is of crown importance. Another question that arises is whether the transport mechanism is concerned with water itself or whether it is mediated by active ion transfer, with water a passive accompaniment. Lastly, the nature of the water exchange in various portions of the cornea is of considerable interest. Since water is diffusing into the cornea from the limbus, presumably the epithelium must be more active in this area than in the center of the cornea.

Dr. Potts deserves tremendous credit for this work, first of all on synthesizing his own tritium and then devising a method of counting it. Those of us who have worked with prepared tritium and elaborate scintillation counters are particularly appreciative of how much work went into this study.

Dr. David M. Maurice (San Francisco): This is very interesting work by Dr. Potts. I am afraid I was unable to take it all in; I was trying to do some rapid calculations, while he was showing the slides.

I don't think it is demonstrated without doubt that there is active transport of water under these circumstances. To start with the delay in the appearance of tritiated water across the beef cornea, about 10 or 20 minutes' delay, I took it to be; I do not find it very surprising because of the slowness of the diffusion of the heavy water in the stroma itself. I found that for radio-sodium in the rabbit cornea, which I suppose is about four times thinner than beef cornea, the delay was about three to five minutes, according to how sensitive the method of detection was. With a stroma four times as thick one would expect a delay 16 times greater, which makes it about 45 minutes; but one must allow for the faster diffusion of water, which makes it about 30 minutes, and there is a difference in temperature, so the difference is not unexpected. About 20 minutes' delay in the appearance time of heavy water is about what one would expect.

Then there is a question of intralamellar injections into the cornea of the rabbit. When you inject between the lamella into the stroma you have approximately half a thickness on each side, which makes the difference between half a thickness of the rabbit's cornea and the whole thickness of the beef cornea, or about eight times, or 64 times faster to reach the surface. So, I don't think there is any difficulty in understanding that. I didn't notice any great difference between the rate of appearance on the epithelial side and the endothelial side when it was injected intralamellarly in the rabbit. I thought the findings were roughly the same at both the endothelial and epithelial surface. If I am wrong I didn't take in that figure. The difference could possibly be due to the fact that one doesn't know at what depth one is injecting the heavy water. There could be a greater thickness of stroma on one side or the other, if the depth of injection was not controlled.

The changes with temperature do not seem to me to be extraordinary. One expects a temperature change of the diffusion rate through the stroma proportional to the change of the viscosity of water with temperature, about 2.5 percent per degree, and for a 30-degree change it is about two to one, I should think. So, one would expect this change to give a two to one change in the diffusion rate.

I think Dr. Potts made a mistake when he said the area of the surface of the rabbit's cornea was 3.14 square cm., and therefore, concluded that the rate of diffusion of heavy water was slower than in Dr. Cogan's results. I think this must be a mistake, because the total area of the rabbit's cornea is only two square cm. There must have been an error in estimating the diameter of the end of the tube.

The details of experimental technique bring up the question of the stirring up of two layers on either side of the cornea. This is very important when there is a rapidly diffusing substance like water. I would like to know how well the two sides of the cornea were stirred.

One general point: Dr. Potts remarked that the sodium mechanism seemed to be ruled out by the fact that there was not a large potential across the endothelium. It seems one would not expect a very big potential difference across the endothelium, since the difference in flux in the two directions, necessary to account for the maintenance of the thickness of the cornea is only about one percent. This would lead to a difference of between 0.5 and 1.0 millivolt, and I believe such a difference was found.

Dr. John E. Harris (Portland, Oregon): Just one question, Dr. Potts. If you calculated your results in terms of the amount of tritiated water per unit volume of water, would not it also have led to this discrepancy, particularly with endothelial damage in the refrigerated specimen?

Dr. V. Everett Kinsey (Detroit): Possibly some of the difference in the results we obtained years ago as compared to Dr. Potts' may be due to species. We used the cat, whose cornea is quite a different thickness from beef. That may possibly account for some of the difference. Perhaps it is in the right direction. Were they faster or slower, Dr. Potts?

Dr. Potts: They were slower.

Dr. Albert M. Potts (closing): I would like to thank the discussers for their very pertinent remarks.

The question of how the fine differences of temperature variation affect the transport, as brought up by Dr. Newell, is certainly work for the future. We stopped at 27°C. because this gave us the maximum transcorneal potential when we measured it and we simply used this temperature as a likely stopping point. That the cornea normally exists at a temperature somewhat lower than 37°C. (just how much lower we don't know) is taken for granted because of the cooling by evaporation and by anterior chamber convection.

The question as to whether the next 10-degree rise in temperature would show us a greater differential is something well worth considering. The greatest amount of differential that we could get would be useful in demonstrating a difference, and if there is a continuous variation with temperature this would add weight to our conclusions.

Work with chemical inhibitors and absence of oxygen is in progress, and we hope this will add additional evidence for mediation by a metabolic process.

As far as the questions Dr. Maurice raised are concerned, a number of them involve misunderstandings. Some of them are fundamental. Dr. Maurice is correct about 3.14 $cm.^2$; the correct figure is 0.8 $cm.^2$

The delay that we emphasized was not the delay in appearance of tritiated water but was the difference in delay between 27°C. and 0°C. That there should be a delay does not surprise us in the least. The delay being so much different with the change in temperature, I do not think, according to the calculations we made, can be accounted for on the basis of difference in viscosity at the two temperatures.

The difference in diffusion rate of tritiated water as compared to ordinary water is something we considered also, and if one uses the approximation of Thovert, that substances in water diffuse at a rate proportional to the square root of their molecular weight, and if for a moment one ignores the various types of hydration of molecules that occur, one calculates a difference of some five percent between tritiated water and normal water, which should not be significant for the purposes of these experiments.

The difference in rate between intralamellar injection and diffusion from one side to the other was not the two times factor that one might expect on the basis of halving the thickness of the cornea, but it was a factor of several orders of magnitude.

There is an additional factor which one introduces when one injects a finite amount of water into the cornea, and that is the actual hydrostatic effect of

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the injection. If this were the whole driving force one would expect a decrease in the rate of water loss as one approaches equilibrium. However, since a decrease in concentration of water retained in the cornea also occurs, it is impossible at the moment to separate the effects of these two types of driving force.

The difference between the amount of water going to the epithelial side and to the endothelial side, I think, is reasonably significant. If we may see slide 3 again I think I can show that there is a difference of several times between the two, particularly if one considers the peak point.

[Slide] The water at room temperature leaving by way of the epithelial side represents some 27 percent of the total dose when considered at this point. The water leaving by way of the endothelial side represents at least twice that, and the differences are increased by lowering the temperature. Again, the difference between 27°C. and 0°C. is the thing we wish to emphasize.

Stirring was accomplished, as in our potential experiments and in Ussing's experiments, by an air lift stirrer which we considered adequate for the purpose. When one puts dye into the chambers and follows it, one can see extremely rapid mixing of the dye caused by the stirrer.

The potential differences across epithelium and endothelium respectively are certainly in a vastly different order of magnitude. If one considers potential difference and "short circuit current" as indicating active transport, then whatever is being actively transported is certainly moving more rapidly across epithelium than across endothelium.

The question by Dr. Harris, concerning the hydration of the cornea, is a pertinent one. I think it is rendered less significant here than in the experiments he was thinking of, because the times of these experiments were relatively short. None of them ran for more than two hours, and the percentage change in weight of the cornea is not great in that period of time. The eyes were equilibrated at room temperature and then the solutions on either side were replaced with solutions at 0°C. and maintained this way by circulating the solution through a cooling bath. So, over the course of two hours, starting from the zero time of the experiment, the corneas were chilled very rapidly. They were not kept for the 24 hours that you used when you studied hydration.

OCULAR CHANGES INDUCED BY POLYSACCHARIDES*

II. Detection of hyaluronic acid sulfate after injection into ocular tissues

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Portland, Oregon

When an hyaluronic acid sulfate is injected into the vitreous, profound ocular pathology results.[1] Usually, the earliest change observed is a liquefication of the vitreous which generally appears a few days after injection. After about a week to 10 days, hydration of the cornea may ensue. The latter is more quickly evident if the material is injected into the anterior chamber.[2] In addition, corneal opacities develop in the anterior portion of the stroma although their appearance is not necessarily associated with antecedent hydration. More delayed effects often include an enlargement of the globe which may or may not be associated with an increase in intraocular pressure. The lens becomes dislocated in many instances and even where this is not evident, the zonules are more susceptible to rupture. Cataracts may develop, and in certain cases a rubeosis and subsequent hyphema have been observed. In short, injection of this substance produces many ocular diseases artificially. The problem is the more intriguing because the acid mucopolysaccharide injected closely resembles certain naturally occurring tissue constituents and it is reasonable to suppose that slight chemical modification of normal composition may produce serious disease.

Elucidation of the mechanism of action of this material requires knowledge both of its chemistry and of the length of time it remains in the eye in its original form. Consideration of available information regarding its chemical structure reveals an impressive similarity to heparin. Thus, as an hyaluronic acid derivative, the hyaluronic acid sulfate

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possesses a basic carbohydrate structure very similar to that of heparin. Furthermore, elemental analysis indicates that the hyaluronic acid sulfate bears three sulfate groups per disaccharide unit while heparin has been reported[3] to contain five sulfate residues per tetrasaccharide unit. The hyaluronic acid sulfate also exhibits biologic properties resembling those of heparin, for example, anticoagulant activity. Heparin, however, exerts only transitory effects, if any, when injected into the eye and thus offers an excellent material to use for control purposes. Such use of heparin makes it desirable, if possible, to distinguish between the hyaluronic acid sulfate and heparin.

Since acid mucopolysaccharides are polyanionic macromolecules and thus migrate in an electric field, it seemed possible that paper electrophoresis under appropriate conditions might provide a rapid and convenient means for separating and identifying these materials. Rienits[4] has found that hyaluronic acid can be readily separated from heparin and chondroitin sulfate by this method in phosphate and acetate buffers of acidic pH. Also, Ujejski[5] has successfully separated various pectic substances by paper electrophoresis in acidic acetate buffers. Even mixtures of neutral di- and polysaccharides can be resolved electrophoretically in borate buffers[6-9] by virtue of the ability of borate to form complexes with certain polyhydroxy compounds.[10] Accordingly, the paper electrophoretic mobility of the hyaluronic acid sulfate as compared with several other acid mucopolysaccharides in buffer systems of varying composition and pH has been investigated.

Two stains widely used for the histologic demonstration of acid mucopolysaccharides in tissue, toluidine blue, and alcian blue have been adapted for locating these materials on the paper strips. Staining of acid mucopolysaccharides with toluidine blue is metachromatic, a phenomenon which is associated with a shift in the absorption maximum of the dye to a shorter wave length. In dilute solutions of toluidine blue, exhibiting only one absorption maximum in the visible range, the location of the new absorption maximum appears to be characteristic of the particular acid mucopolysaccharidee complexing with toluidine blue.[11] Therefore, spectrophotometric study of the reaction with toluidine blue of materials obtained from treated eyes, either before or after paper electrophoresis, might provide further evidence to aid in identifying the material in question. Although staining of acid mucopolysaccharides with alcian blue is not metachromatic, the reaction was studied spectrophotometrically in an attempt to find other useful criteria for identifying various acid mucopolysaccharides.

Methods

Paper electrophoresis was carried out in the Beckman/Spinco Model R apparatus using Schleicher and Schuell 2043A-mgl strips and Schleicher and Schuell 470 filter paper wicks. When a given substance was applied to the strips at either end of the apparatus, it migrated more slowly than when applied to any of the six inner strips. For this reason, the two end strips were not used when rates of migration were compared. Staining was carried out in Spinco staining racks and trays.

All acid mucopolysaccharides studied thus far were commercial preparations* The sulfated acid mucopolysaccharides (hyaluronic acid sulfate, heparin, carrageenan, chondroitin sulfate) were applied to the strips in approximately 30 μg. quantities (15 μl. of a solution containing 2.0 mg./ml.) and the carboxylic acid mucopolysaccharides (hyaluronic acid, pectin) in approximately 60 μg. quantities (15 μl. of a solution containing 4.0 mg./ml.). At the end of a run, the strips

* Hyaluronic acid sulfate (SN126) was supplied through the courtesy of Dr. Robert L. Craig of G. D. Searle and Company and carrageenan (Sea Kem 21) through the courtesy of Leonard Stoloff of Seaplant Corporation. Heparin was obtained from Darwin Laboratories; chondroitin sulfate, hyaluronic acid, and pectin, N.F. from Nutritional Biochemicals Corporation.

were dried in an oven at 120 to 125°C. for 10 to 15 minutes. Drying time had to be carefully controlled when phosphate buffer was used since prolonged exposure to heat apparently resulted in the formation of material (s), probably polyphosphates, which take the acid mucopolysaccharide stains and thus interfere with the detection of acid mucopolysaccharides.

In many experiments, aliquots of a given material were applied to duplicate strips, one of which was stained with alcian blue and the other with toluidine blue. All the acid mucopolysaccharides studied took the alcian blue stain but the samples of hyaluronic acid and pectin obtained failed to stain with toluidine blue, under the conditions employed. Although hyaluronic acid and pectin were detectable with alcian blue in rather small quantities, still smaller quantities of the sulfated acid mucopolysaccharides were revealed by this stain. Toluidine blue appeared to be even more sensitive, however, for those materials taking that stain.

Toluidine blue stain. The strips were immersed in toluidine blue solution (0.01 percent in 0.5-percent acetic acid) for one minute[12] followed by three rinses of five minutes' duration in two percent acetic acid.

Alcian blue 8 GS stain. The strips were immersed in alcian blue solution (0.1 percent in 3.0-percent acetic acid, pH 2.5)[13] for 30 minutes[14] followed by three rinses of five minutes' duration in five-percent acetic acid. These three acetic-acid rinses reduce background color in the paper strips very appreciably.

All stained strips were dried in air.

The following buffers were used. Before use, the pH of each buffer was checked with a Beckman Model G pH meter.

1. Barbital buffer, pH 8.6, the buffer routinely used for paper electrophoresis of serum proteins.

2. Borate buffers, approximately 0.1 M. Varying proportions of 0.20 M. H_3BO_3 and 0.20 M. NaOH were mixed and diluted to one liter.

3. Acetate buffers, approximately 0.1 M. Prepared in a similar manner to the borate buffers from 0.20 M CH_3COOH and 0.20 M CH_3COONa.

4. Phosphate buffers, 0.1 M. Different volumes of 0.20 M Na_2HPO_4 and 0.20 M KH_2PO_4, which always gave a total volume of 500 ml., were mixed, then diluted to one liter.

Solutions for spectrophotometric studies of the metachromatic reaction of toluidine blue with the acid mucopolysaccharides were prepared as follows:

Toluidine blue, 2×10^{-5} M in water (dye content of the reagent was taken into account in calculating the amount to use) in 5.0 ml. aliquots was mixed with 1.0 ml. aliquots of solutions of the acid mucopolysaccharides containing 0.20 mg./ml., or with 1.0 ml. of distilled water for the toluidine blue spectrum. All samples were read in a Beckman Model DU quartz spectrophotometer against distilled water blank.

Spectrophotometric studies of the reaction with alcian blue were carried out in a similar manner using 0.005 percent alcian blue in distilled water. Molar concentrations of alcian blue cannot be given, since the molecular structure of the dye is not accurately known.[15, 16]

Results

Barbital buffer. Both the hyaluronic acid sulfate and heparin migrated much more rapidly than serum albumin in this buffer. When current was applied as recommended for the separation of serum proteins (2.5 ma. for 16 hr.) the acid mucopolysaccharides were detectable only at the extreme ends of the strips. Current conditions were modified (5.0 ma. for 6.0 hr.) so that the acid mucopolysaccharides remained on the strip, but the bands obtained with this buffer were quite diffuse and irregular, making it seem advisable to seek a better medium. Although the bulk of the hyaluronic acid sulfate preparation migrated together, noticeable trailing of metachromatic material from the origin

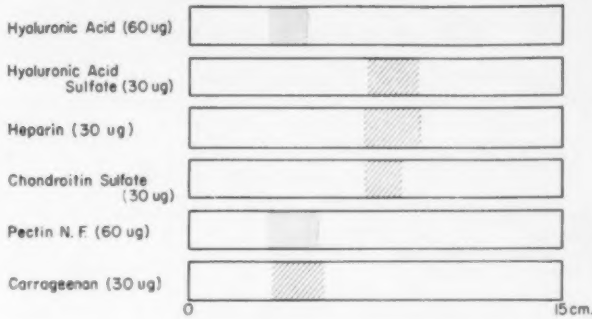

Fig. 1 (Talman and Harris). Electrophoretic migration of acid mucopolysaccharides on paper in 0.10 M borate buffer, pH 11.7. Current flow, 4.0 ma., four hours.

was observed indicating that the preparation was not a pure compound.* Such trailing of metachromatic material was minimal with the heparin preparation.

Borate buffers. Earlier studies on the electrophoretic separation of neutral carbohydrates in borate buffers employed solutions varying in pH from 9.2 to 11.[6-9] Attempts to separate the acid mucopolysaccharides in borate buffers of pH less than 11 were unsuccessful. For the most part, any acid mucopolysaccharide detected after paper electrophoresis in such buffers appeared as longitudinal streaks. In borate buffers of pH 11.7 to 12.2 (corrected for Na+ effect), however, most of the hyaluronic acid sulfate and heparin appeared as distinct bands with even fronts after the application of four milliamperes constant current for four hours. Results of a typical experiment are illustrated in Figure 1. There was again noticeable trailing of metachromatic material from the origin with the hyaluronic acid sulfate, while such trailing was minimal with heparin.

Hyaluronic acid, heparin, and the hyaluronic acid sulfate all have a basic carbohydrate structure of the glucuronidoglucosaminidic type.[17] The former is a carboxylic acid mucopolysaccharide while the two latter are sulfated mucopolysaccharides which appear to differ in sulfate content by only one sulfate group per tetrasaccharide unit. At the high pH required for satisfactory migration of these materials in borate buffer, these sulfate groups should be completely ionized and it is, therefore, not surprising to find heparin and the hyaluronic acid sulfate migrating at essentially the same rate. There was, however, a clear-cut separation of these two acid mucopolysaccharides from hyaluronic acid.

Chondroitin sulfate, which differs qualitatively from the hyaluronic acid type of mucopolysaccharide in containing galactosamine rather than glucosamine and which bears one sulfate group per disaccharide unit, migrated more slowly and in a somewhat more discrete band than heparin and the hyaluronic acid sulfate, but the separation was not definitive. This small difference in electrophoretic behavior might aid in identifying purified preparations, however. Clear-cut separation of chondroitin sulfate and hyaluronic acid was achieved.

Pectin, which is also a carboxylic acid mucopolysaccharide, behaved quite similarly to hyaluronic acid in this buffer even though it is composed of galactose derivatives. Pectin resembled carrageenan in paper electrophoretic behavior even more closely, however. The latter is a sulfated mucopolysaccharide extracted from the red sea plants, Irish moss or Carrageen, and is composed of sulfated D-galactose and anhydro-D-galactose units. Pectin and carrageenan cannot be distinguished from each other nor from hyaluronic

* Study of the hyaluronic acid sulfate preparation in the analytical ultracentrifuge revealed no sharp boundary.[18]

TABLE 1

EFFECT OF PH OF PHOSPHATE BUFFER (0.1 M) ON THE PAPER ELECTROPHORETIC SEPARATION OF SEVERAL ACID MUCOPOLYSACCHARIDES

(4.0 ma. constant current applied for four hr.)

Acid Mucopolysaccharide	0.1 M Phosphate Buffer			
	pH 6.0 Band Limits*	pH 6.2 Band Limits	pH 6.4 Band Limits	pH 6.6 Band Limits
Hyaluronic acid	0.4–3.5	0.2–3.3	0.5–3.2	0.6–3.2
Heparin	5.3–6.5	5.5–6.6	4.9–6.3	4.7–6.0
Hyaluronic acid sulfate	5.2–6.2	5.1–6.0	4.9–6.3	4.6–5.7
Chondroitin sulfate	4.8–5.6	4.7–5.6	4.5–5.6	4.3–5.3

* Distance of trailing and leading edges respectively, of main band from point of application.

acid by this means but may be readily distinguished from the hyaluronic acid sulfate, heparin, and chondroitin sulfate. These results suggest that the paper electrophoretic behavior of the acid mucopolysaccharides in borate buffer is influenced by the acidic groups as well as the configuration of the carbohydrate chain and that such studies might be of value in studying the structure of acid as well as neutral polysaccharides.

Acetate buffers. Ujejski[5] utilized acetate buffers for the paper electrophoretic separation of pectic substances but with the acid mucopolysaccharides studied in this series of experiments, electrophoresis in acetate buffers (pH 4.1, 4.9, 5.4, 5.9, 6.4, 6.9 at 4.0 ma. for 4.0 hr.) yielded only diffuse bands with curved fronts. Heparin and most of the hyaluronic acid sulfate migrated at the same rate (the hyaluronic acid sulfate again displayed trailing of metachromatic material from the origin) and pectin and carrageenan again migrated somewhat more slowly. The acetate buffers offered no advantages over borate buffer for separating acid mucopolysaccharides of very similar structure and actually proved inferior to borate buffer from the standpoint of obtaining well-defined bands of material.

Phosphate buffers. The applicability of phosphate buffers to the paper electrophoretic separation of acid mucopolysaccharides was investigated using a series of 10 buffers ranging in pH from 4.5 to 7.7. After paper electrophoresis in all these buffers, including the one having an alkaline pH, the more highly purified acid mucopolysaccharides appeared as well-defined bands with even fronts. The less pure preparations did not appear as narrow bands but did display even fronts of migration. As with the buffers previously studied, the hyaluronic acid sulfate and heparin were difficult to separate but showed small differences in rate of migration around pH 6.0.

Examination of Table 1, which summarizes these results, indicates that a phosphate buffer of pH 6.2 probably offers the best medium for separating the hyaluronic acid sulfate and heparin, both from the standpoint of slightly different rates of migration and of presenting the materials in discrete bands.

Optimum conditions of current were investigated using the phosphate buffer of pH 6.2. These results are summarized in Table 2. Using 4.0 ma. constant current for six hours, heparin migrated approximately 0.6 cm. further than the hyaluronic acid sulfate. Increasing the current intensity to 5.0 ma. obliterated most of the difference in rates of migration of the hyaluronic acid sulfate and heparin and broadened the bands somewhat. Holding the current intensity at 4.0 ma. but extending the time interval to eight hours, produced similar results. Reducing current intensity to 3.0 ma. and applying it for a period of eight hours (giving the same total current as with 4.0 ma. for 6.0 hr.) gave rates of migration similar to those obtained with

TABLE 2

EFFECT OF ALTERING CONDITIONS OF CURRENT FLOW ON THE PAPER ELECTROPHORETIC SEPARATION OF ACID MUCOPOLYSACCHARIDES IN 0.1 M PHOSPHATE BUFFER pH 6.2

Acid Mucopolysaccharide	Current Intensity and Duration of Run			
	4 ma.–6 hr. Band Limits*	5 ma.–6 hr. Band Limits	3 ma.–8 hr. Band Limits	4 ma.–8 hr. Band Limits
Hyaluronic acid	0.2–3.3	0.8–4.4	0.4–3.5	1.0–4.6
Heparin	5.5–6.6	6.2–7.7	5.2–6.7	6.7–8.4
Hyaluronic acid sulfate	5.1–6.0	6.5–7.6	5.1–6.2	7.0–8.4
Chondroitin sulfate	4.8–5.6	5.7–6.9	4.3–5.3	6.3–7.5

* Distance of trailing and leading edges, respectively, of main band from point of application.

4.0 ma. for six hours. The bands appeared to be somewhat more diffuse, however, the latter observation, coupled with the fact that the six-hour time interval is technically more convenient has led to the adoption of 4.0 ma. for six hours as the conditions of current flow for separation of these acid mucopolysaccharides.

Results of a typical experiment employing these conditions are illustrated in Figure 2. Here it can be seen that hyaluronic acid migrates sufficiently more slowly than the hyaluronic acid sulfate, heparin, and chondroitin sulfate to permit a clean separation of the former from any one of the latter three by this means. Chondroitin sulfate can be easily distinguished from the hyaluronic acid sulfate and heparin on separate strips because of its slower rate of migration but mixtures of chondroitin sulfate and heparin or chondroitin sulfate and the hyaluronic acid sulfate on the same strip were not resolved. Results obtained with hyaluronic acid, heparin, and chondroitin sulfate in this series of experiments are similar to those previously reported by Rienits.[4]

SPECTROPHOTOMETRIC STUDIES

Results obtained with toluidine blue and acid mucopolysaccharides in solution are summarized in Table 3. The sulfated acid mucopolysaccharides showed the expected[11] shifts in absorption maxima to shorter wave lengths in the visible region and also at one peak in the ultraviolet region. Hyaluronic acid, however, produced only very minor

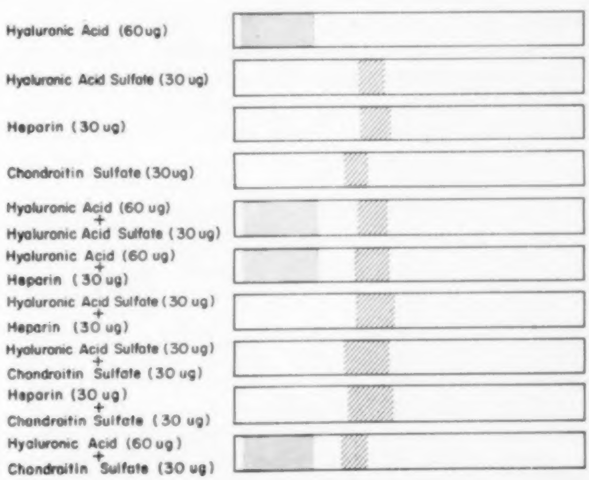

Fig. 2 (Talman and Harris). Electrophoretic migration of acid mucopolysaccharies and mixtures of acid mucopolysaccharides on paper in 0.10 M phosphate buffer. Current flow, 4.0 ma., six hours.

TABLE 3

ABSORPTION MAXIMA AND MINIMA OF TOLUIDINE BLUE DYE ALONE AND MIXED WITH VARIOUS ACID MUCOPOLYSACCHARIDES*

	Maxima			Minima
Toluidine blue	244	290	636–637	253
Toluidine blue +hyaluronic acid sulfate	245	273	534	255
Toluidine blue +heparin	245	273	531–533	254
Toluidine blue +hyaluronic acid	244	287	638	253
Toluidine blue +chondroitin sulfate	246	276–77	547	255

* 5.0 ml. of 2.0×10^{-5} M toluidine blue mixed with 1.0 ml. of distilled H_2O for the toluidine blue spectrum and with 1.0 ml. of acid mucopolysaccharide solutions containing 0.20 mg./ml. for spectra of the complexes.

changes in the absorption spectrum of toluidine blue. The latter observation correlates with the fact that this particular hyaluronic acid preparation was nonmetachromataic. Although these absorption maxima do not appear as sharp peaks, they are reproducible and the location of the absorption maximum of chondroitin sulfate + toluidine blue is sufficiently different from those of heparin + toluidine blue and hyaluronic acid sulfate + toluidine blue to be of value in distinguishing the former from the latter two. The heparin and hyaluronic acid sulfate complexes with toluidine blue exhibit only minor differences in absorption spectrum but the slight difference in location of the maximum in the visible region might be of some value in distinguishing between the two when used in conjunction with other data.

Results of similar studies carried out with alcian blue 8 GS are summarized in Table 4. Dye complexes with the sulfated acid mucopolysaccharides again exhibit a shift in the absorption maximum to a shorter wave length at the peak in the visible region. The chondroitin sulfate complex showed a slight shift toward the shorter wave length at the peak in the region of 330 mμ. Otherwise changes in the absorption spectrum of alcian blue as a result of forming complexes with acid mucopolysaccharides were negligible. The hyaluronic acid-alcian blue complex failed to exhibit a significant change in absorption spectrum even at the peak in the visible range and shifts observed with the sulfated acid mucopolysaccharides were small (10 to 12 mμ).

It is not surprising to find such small changes in absorption spectrum in view of the fact that this dye stains acid mucopolysaccharides a darker shade of the same color, rather than staining metachromatically. Also, fresh solutions of the alcian blue-acid mucopolysaccharide complexes often exhibit greater extinctions than alcian blue mixed with the same volume of water.

Changes in the light absorption of these solutions must be interpreted cautiously, however, since a precipitate separates from them upon standing. The exact structure of alcian blue is unknown, but a brief description of its preparation[15] indicates that the molecule may well contain quaternary am-

TABLE 4

ABSORPTION MAXIMA AND MINIMA OF ALCIAN BLUE 8 GS ALONE AND MIXED WITH VARIOUS ACID MUCOPOLYSACCHARIDES*

	Maxima			Minima	
Alcian blue 8 GS	257	332–334	616–617	243	302
Alcian blue 8 GS+hyaluronic acid sulfate	259	330–333	607–609	242–243	302
Alcian blue 8 GS+heparin	259	330–333	606–607	242	302
Alcian blue+hyaluronic acid	258	333–334	615–617	243	304
Alcian blue 8 GS+chondroitin sulfate	257	328	604–605	243	302–304

* 5.0 ml. of 0.005 percent Alcian blue mixed with 1.0 ml. of distilled H_2O for the alcian blue spectrum and with 1.0 ml. of acid mucopolysaccharide solutions containing 0.20 mg./ml. for spectra of the complexes.

monium groupings and Scott has shown that acid mucopolysaccharides are precipitated by quaternary ammonium salts such as cetylpyridinium chloride.[18] Thus, the staining of acid mucopolysaccharides by alcian blue may be based upon the precipitation of the former by the latter with the color of the dye rendering small amounts of precipitate easily visible.

Scott has also shown[19] that, under specified conditions, complexes of carboxylic acid mucopolysaccharides with quaternary ammonium ions are more soluble than such complexes of the sulfated acid mucopolysaccharides. The weaker staining with alcian blue of the carboxylic acid mucopolysaccharides in comparison to the sulfated acid mucopolysaccharides may provide another example of this phenomenon, or the ionization of the carboxylic groups may be sufficiently suppressed by the low pH used for the dye solution in order to increase its specificity for acid mucopolysaccharides to impair formation of the dye complex. The latter possibility has not been ruled out.

Although toluidine blue is not a quaternary ammonium compound, it is a cationic dye and thus should form complexes with the anionic acid mucopolysaccharides similar to those formed with quaternary ammonium compounds. Blumenkrantz has reported[20] that mixtures of toluidine blue and acid mucopolysaccharides do form precipitates. Such complexes are probably more soluble than those formed with alcian blue, however, since precipitates were never visible to the naked eye in the solutions used for the spectrophotometric studies reported here even after standing for 24 hours.

Comment

These studies show that the hyaluronic acid sulfate and heparin cannot be unequivocally distinguished from each other either by electrophoresis or by spectrophotometric study of their toluidine blue and alcian blue complexes. They may be readily distinguished from the commercial hyaluronic acid employed by both methods, however, and preliminary studies indicate that the hyaluronic acid present in the vitreous humor behaves in a manner similar to that of the commercial sample. Furthermore, the concentration of hyaluronic acid in the vitreous humor is so low that it is not detected on paper electrophoretic strips by these techniques and so does not present a problem in any case. Therefore, demonstration of the presence of a material migrating at the same rate as known hyaluronic acid sulfate or heparin in an eye previously injected with the preparation in question should provide good presumptive evidence for the continued presense of the injected material.

In this way, paper electrophoretic studies may be utilized to estimate how long the hyaluronic acid sulfate or heparin remains in the eye. Phosphate buffer (0.1 M, pH 6.2) seems best suited for such studies, since heparin appears to migrate a little faster than the hyaluronic acid sulfate under these conditions, although borate buffer might also be used. Apropos of borate buffers, results obtained in this series of experiments suggest that electrophoresis in such buffers might be of value for studying the structure of acid as well as neutral polysaccharides.

As this investigation progressed, the similarity of the hyaluronic acid sulfate to heparin became increasingly clear. On this basis it may be postulated that the minor chemical and structural differences between the two may hold the key to the deleterious effects of the hyaluronic acid sulfate. However, the validity of such an hypothesis cannot be established and the mechanism of action of the material cannot be elucidated until the component or components responsible for the effects of this rather impure preparation have been identified. Nevertheless, even in its present form the hyaluronic acid sulfate is a tool of real value since a means for producing pathologic conditions at will greatly expedites study of the physiologic and metabolic changes associated with them.

Finally, the hazard involved in assigning

any physiologic significance to histologic studies with such dyes as alcian blue, toluidine blue, or others which presumably identify acid mucopolysaccharides should be emphasized. Not only are these techniques not specific acid for mucopolysaccharides but, more important, many of the compounds giving the same positive stain by virtue of their similar chemical properties often possess profoundly different biologic activities.

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Discussion

Dr. Jin H. Kinoshita (Boston): This is a very interesting compound in that it is probably the most highly sulfated compound polysaccharide available. It is not found normally in tissues.

It would be interesting to study its metachromatic staining properties, how much the extra sulfate groups contribute, and also perhaps make hydration studies similar to those made by Dr. Smelser.

If the compound can be obtained in the pure state I believe it would be quite interesting to find out fundamental things about mucopolysaccharide.

Dr. Seymour P. Halbert (New York): I would like to ask one question about the source of the hyaluronic acid and whether it was analyzed for the presence of any residual protein; small amounts of residual proteins which may have toxic properties could conceivably produce some of the lesions shown.

Another question is whether the author injected hyaluronic acid alone, without sulfation into the eye, and what happened then.

The reason I raise this question is because for some years we have been growing beta hemolytic streptococci in connection with other work, and as a by-product we have produced large amounts of hyaluronic acid which have been highly purified and studied by Dr. Karl Meyer. It has been found to be extremely homogeneous, of high molecular weight, and extraordinarily stable. We have had a solution, a highly viscous solution, in the icebox for three years without any evidence of depolymerization.

This raises the possibility that, if hyaluronic acid alone is nontoxic, it might conceivably be used in the treatment of retinal detachment, because when injected it possibly could reside in the vitreous and achieve the same purpose as does vitreous implantation. I understand there is very little, if any, hyaluronidase in ocular tissues, and such an extremely stable and highly purified hyaluronic acid might act as a suitable substitute for this purpose.

Dr. Ellen Talman (closing): The preparation which we used was received through the courtesy of the G. D. Searle Company, and the material was prepared from a purified umbilical cord hyaluronic acid. With regard to the protein content, I have not done any studies particularly designed for that. However, when one stains a paper electrophoretic strip to which this material has been applied, staining for protein, one finds no indication whatsoever of protein.

We have not tried injecting hyaluronic acid into the eye. I will have to leave the discussion of clinical implications to my clinical colleagues.

ELEVATION OF CHOROID BY INSERTION OF POLYVINYL SPONGE*

An experimental study

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Several operations for retinal detachment displace the choroid inward so that the retina may more easily fall against it, to be bound to it by the reaction to diathermy. One of these operations, lamellar scleral resection,[1] decreases the area of the surface of the globe, and the inward buckling of the deep scleral lamellae pushes the choroid inward.[2, 3] In the scleral buckling operation of Schepens[4] both the sclera and the choroid are displaced inward by a plastic tube. The Everett scleral-fold operation[5–7] and the application of surface diathermy to the sclera are other methods of decreasing the circumference of the eyeball.

A different principle for approximating the choroid to the retina was reported by Strampelli[8] in 1954. He inserted a gelatin sponge into the suprachoroidal space through a single scleral incision, after separating the choroid from the sclera with a spatula. The sponge was gradually absorbed, so that the elevation of the choroid that it produced was temporary.

The use of a more durable material than gelatin to elevate the choroid, to our knowledge, had never been explored. Such a material, to have clinical value, would have to possess certain chemical and physical properties:

1. It should be chemically inert so that it would incite only minimal cellular reaction within the eye.

2. It should be able to maintain its size and shape within the eye for an indefinite period.

3. It should be easily sterilized, shaped, and inserted into the eye. Although there has been a natural reluctance through the years to insert any foreign material within the eye because of the risk of sympathetic ophthalmitis, one of us (J. W. H.) was aware of a solid material, used for some less refined experimental work[9] than that which we contemplated, that might be suitable for such a project and we decided to try it. This material, polyvinyl sponge, when inserted into the vitreous chamber of dogs' eyes, had proved to be relatively innocuous.

Material

Polyvinyl sponge (ivalon) is a polymer of polyvinyl alcohol with formaldehyde. It is fine textured, and, when wet, is soft, flexible, elastic, and easily cut with scissors. It can be sterilized by boiling without altera-

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tion. In the body it acts as a framework for the formation of fibrous tissue,[10, 11] and it has been used to occupy space after pneumonectomy,[10] as an orbital implant,[12] and in repair of such lesions as aortic aneurysms,[13] cardiac aneurysms,[14] cardiac septal defects,[15] and defects of the chest wall.[16]

METHOD

For our study polyvinyl sponge was implanted into 27 eyes of 27 dogs (dogs 1 to 27); the other eye of each dog was used as a control. The first 18 operations were designed primarily to demonstrate the reaction of the eye to the foreign material. For these, a standardized procedure was used, and in order to cause as little surgical trauma as possible, only thin strips of the sponge, ranging from 1.5 to 3.5 mm. in thickness, were used. The other nine operations were done to determine the size of implants which it is feasible to introduce into the suprachoroidal space, the accessibility of different parts of the eye for this operation, the effect of using sponge compressed before its insertion, and the variations in the surgical technique that could be employed to insert the sponge. Diathermy was not used, nor were artificial retinal detachments produced, as these would have tended to obscure the basic reaction to the sponge.

The eyes were examined ophthalmoscopically. The animals were killed at intervals ranging from 24 hours to 59 weeks after the operations. The eyes were enucleated and examined in the gross, before and after being opened. Microscopic sections were made of 22 of the eyes in which sponge had been implanted. The remaining five eyes were saved for demonstration of their gross characteristics.

The technique for the first 18 operations was as follows: The polyvinyl sponge was washed for one hour in running water and was then sterilized by boiling in distilled water for 30 minutes. The water was pressed out of the sponge, and it was measured prior to its insertion. In the first 18 operations the strips of sponge were usually 3.0-mm. wide, but a few were up to 5.0-mm. wide. Length varied from 5.0 to 23 mm. Larger sizes were used in some of the later operations.

The dogs were anesthetized with pentobarbital sodium given intravenously. The conjunctiva and bulbar fascia were incised to expose the superior part of the sclera. In most cases, the superior rectus muscle was severed to give better access to the sclera, and it was re-attached at the end of the operation with 4-0 black silk sutures.

Two anteroposterior scratch incisions through the sclera were made with a Graefe knife. Their anterior ends corresponded to the location of the ora serrata, and they were made long enough to accommodate the width of the sponge selected. Their separation from each other depended on the length of the sponge to be implanted. The choroid between the two incisions was separated from the sclera with a spatula used for cyclodialysis, or with an iris repositor which was curved to match the contour of the globe.

Paracentesis of the anterior chamber was done in 26 of the 27 dogs (dogs 1 to 27; exception dog 23) in order to prevent excessive rise in the intraocular pressure when the sponge was inserted.

An iris repositor with a flexible blade and a small hole drilled through it near the tip of the blade was shaped to correspond to the curvature of the globe. The blade was passed into the suprachoroidal space through one incision, to emerge from the other. One end of the sponge was tied to the emerging tip of the iris repositor (fig. 1*a*), which was then drawn back, pulling the sponge after it into the suprachoroidal space (fig. 1*b*). The sponge was trimmed flush with the sclera with scissors, and its ends were tucked in away from the lips of the incisions with a spatula. The scleral incisions were closed with from one to three sutures of 5-0 black silk (fig. 1*c*), and the conjunctiva was closed with catgut.

The technique for the other nine dogs varied. The technique of pulling the sponge

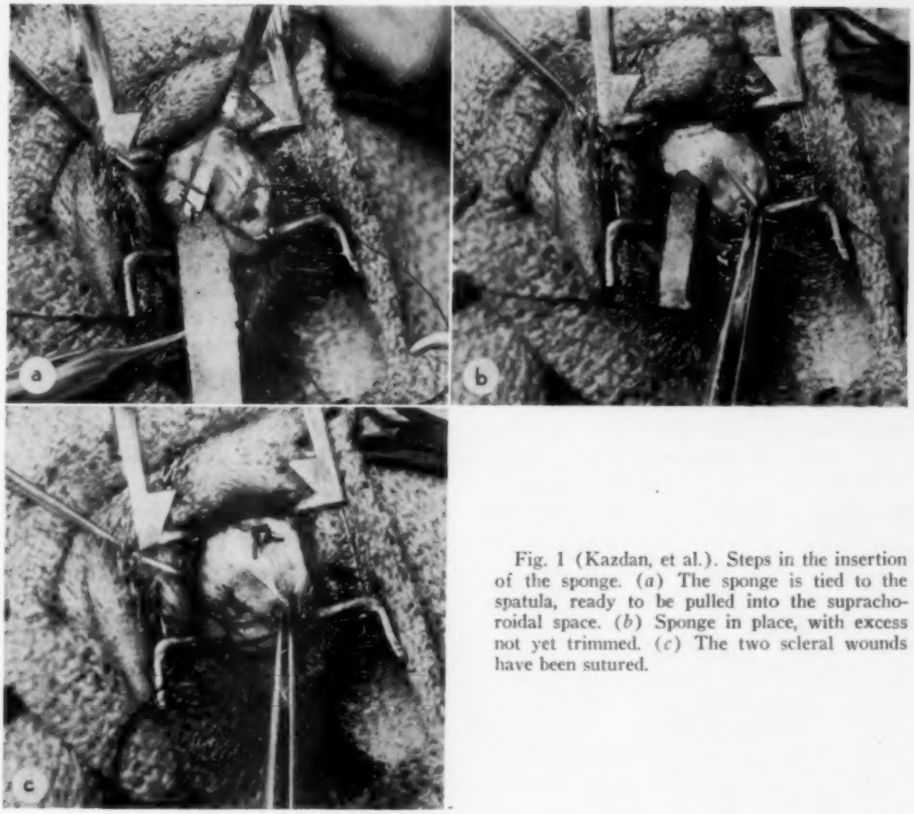

Fig. 1 (Kazdan, et al.). Steps in the insertion of the sponge. (*a*) The sponge is tied to the spatula, ready to be pulled into the suprachoroidal space. (*b*) Sponge in place, with excess not yet trimmed. (*c*) The two scleral wounds have been sutured.

through the suprachoroidal space from one incision to another was used for seven eyes. In one of these operations the two scleral incisions were made parallel to the limbus, rather than perpendicular to it (dog 22). In another eye (dog 10), a large piece of sponge, 8.0 by 6.0 by 3.5 mm., was implanted near the posterior pole of the eye to demonstrate the accessibility of this region for this procedure. Another variation was the implantation of a single, long (23 mm.) strip of sponge entirely across the two upper quadrants of the eye, by the use of three, rather than two, scleral incisions (dog 16). The sponge was first drawn into the eye from the 9- to the 12-o'clock incision. The long emerging end was then drawn through the suprachoroidal space from the 12- to 3-o'clock positions.

A different method was used for the other two eyes. In these, a single scleral incision was made parallel to the limbus over the ora serrata. The choroid was separated from the sclera posterior to the incision with a spatula, and the sponge was pushed into position with the spatula and fine forceps. The sponges used were relatively large; one was 15 by 5.0 by 3.5 mm., and the other was 8.0 by 4.5 by 3.5 mm. They were introduced without difficulty, but at the time of operation it was not possible to tell whether or not they lay flat or were curled.

Various-sized pieces of sponge were inserted to explore the limits of size which might be used with safety and ease. The largest piece used was 9.5-mm. long, 10 mm. wide, and 8.0-mm. thick.

Due to the effect of the tissue pressure, the elevation produced by the sponge was less than the thickness of the sponge prior to its insertion when the eyes were open. In order to decrease this difference, a pre-compressed sponge was used in one animal (dog 9). The sponge was compressed from an original thickness of 10 to 3.0 mm. by a smooth-faced metal clamp, and was boiled while in the clamp. When the sponge cooled and the clamp was removed, it retained its compressed form. The size of the implanted piece was 20 by 5.0 by 3.0 mm.

The animals were killed by intravenous injection of pentobarbital sodium, and the eyes were removed immediately. The eyes were hardened in 10-percent formalin for at least 24 hours, then were washed in water, and placed in alcohol. They were examined grossly, and opened. The opened (capped) eyes were dehydrated, embedded in paraffin, and sectioned. Staining was with hematoxylin and eosin by the techniques used for human eyes. Vernier calipers were used to measure the dimensions of the sponge in the gross specimens and on the slides.

Postoperative course

Healing was rapid, and in animals kept alive more than a month, the eye was white. The exception was dog 24, in which the conjunctiva was red 13 months after operation, and in which an inflammatory reaction was found in the sponge. The anterior chambers quickly regained their normal depth, and light reflex was present in all eyes.

Ophthalmoscopically, the media remained clear in most of the eyes, and the posterior poles looked normal in all but one. Sponges that were 1.5-mm. thick caused no ophthalmoscopically visible elevation. Some of the thicker sponges produced elevations of the retina and choroid, but they were so far anterior that the amount of elevation could not be measured well. Disturbance of pigment or retinal and choroidal holes were seen with the ophthalmoscope in 11 of the 12 eyes in which these defects were found later on gross examination of the opened eye. These lesions were usually at the ends of the sponge, and none were distant from the position of the sponge.

In one dog (dog 12), choroidal separation was observed in the immediate postoperative period. However, the fluid was absorbed, and there was no sign of choroidal separation when the eye was enucleated at eight weeks, although a small choroidal scar was observed.

Dog 21 had a vitreous opacity and a retinal hemorrhage above the disc. At operation some vitreous had been withdrawn by a hypodermic needle inserted through the pars plana. The hemorrhage absorbed, but a vitreous band attached to the scar of the perforation was found when the eye was opened at 50 weeks.

In one dog (dog 27) a retinal hemorrhage in the region of the sponge and a small vitreous hemorrhage were observed in the early postoperative period. No vitreous had been lost at operation. At the time of enucleation, 59 weeks after operation, the vitreous was clear, and the hemorrhage had been absorbed.

Gross examination

The gross appearance of the unopened eyes was normal in most cases except for the presence of the silk sutures and the small scars of the paracentesis. All the scleral wounds were healing well or were healed, and in no case was the sponge extruded.

In four eyes there was a mass of tissue on the surface of the globe in the region of the insertion of the superior rectus muscle. One of these was the eye which had an inflammatory reaction at 56 weeks (dog 24) but the other three had the expected histologic pictures, although there were small retinal holes in two of them.

The opened eyes. Sponges which were only 1.5 mm. thick before insertion produced little or no elevation which could be seen on inspection of the retina. If a section was made through the sponge, it could be seen grossly and measured. Sponges which were 3.0 mm.

or more thick before insertion produced elevation of retina and choroid which had sloping margins (fig. 2a).

In all eyes the retina appeared intact and normal over most of its area, including the part over the sponge. Small retinal folds over the sponge were present in the eyes examined at 24 hours, five and seven days, and four weeks after operation. A small hemorrhage was present in the base of one of the folds in the eye examined 24 hours after operation.

In seven eyes there were areas over, or at the ends of, the sponge which looked like areas of healed choroiditis of human eyes. The usual size of such an area was 1.0 mm., only one or two were present in an eye, and none exceeded 2.0 mm. in size. In 11 eyes one or two holes were observed in the retina and choroid, usually at the ends of the sponge, under the scleral sutures. These holes were usually about 1.0 mm. in diameter, but one (dog 27) was about 2.5 mm. in diameter, and another (dog 6) was 3.3 mm. in diameter and exposed the underlying sponge. At the margins of these holes the retina was bound to the underlying tissue, and in none of the eyes was the retina detached.

The vitreous was normal in 21 of the 27 eyes. In each of the four others (dogs 18, 21, 22, and 26) the vitreous contained one or more fine white or translucent bands which were attached to a retinal hole. One band ran to the back of the lens, and another to the posterior part of the elevation produced by

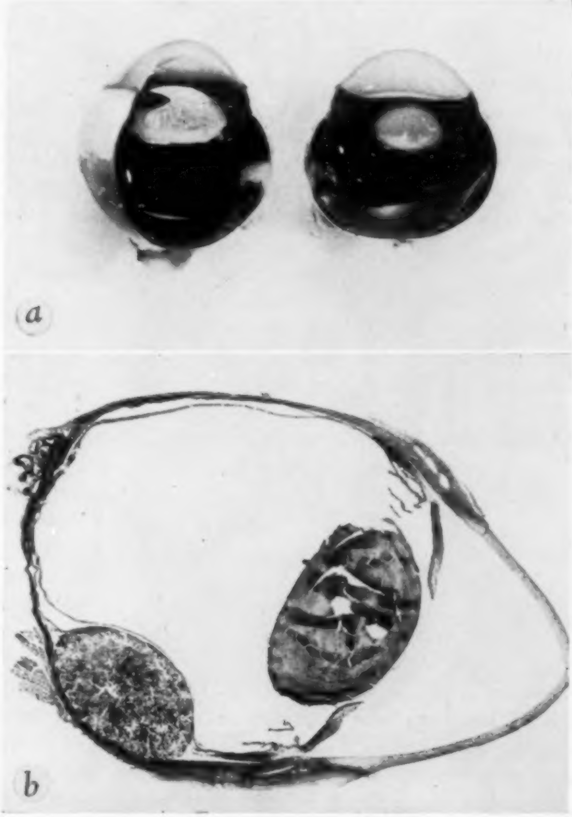

Fig. 2 (Kazdan, et al.). Dog 21. Fifty weeks after operation. (*a*) The eye has been opened in the sagittal plane through the sponge. The choroid is elevated 3.4 mm. by the sponge. (*b*) Dog 21. The lens-like shape of the sponge shown in (*a*) in section, and the elevation of the choroid and retina. (Hematoxylin-eosin, ×4.)

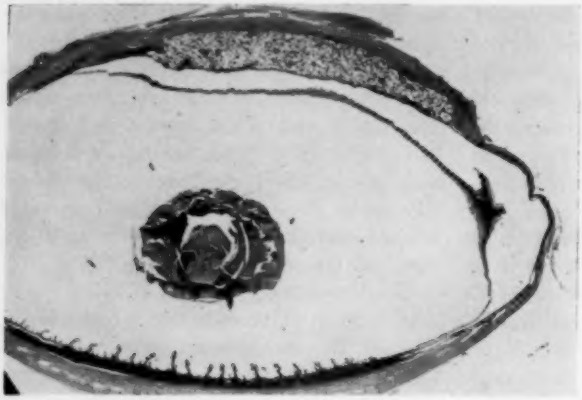

Fig. 3 (Kazdan, et al.). Dog 8. View of an approximately frontal section in the region of the ora serrata five weeks after operation, showing the attachment of the sponge to the sclera and the elevation of a large extent of the choroid. (Hematoxylin-eosin, ×4.)

the sponge. In two other eyes (dogs 14 and 15) some condensation of the vitreous had occurred in the region of the implant but the retina was not disturbed.

The anterior chamber, iris, ciliary body, and optic disc were normal in all eyes.

Elevation of the choroid and retina by the sponge. In sagittal section the sponge was tapered at its anterior and posterior ends; its outer edge conformed to the curvature of the sclera (figs. 2*a* and 2*b*), except in several cases in which the sclera bulged outward, and its inner surface pushed the choroid and retina inward. Measurements were made of 19 implants. The thickness of the implant in situ in the eye was 30 to 85 percent less than its thickness before insertion and averaged about 50 percent of the original thickness. The decrease in thickness was not related to the interval since operation. The compressed sponge, which was 3.0 mm. thick at operation, at five weeks after operation was 2.3 mm. thick in the eye, or about three fourths of its original thickness (fig. 3).

Microscopic examination

All the sponges were between the sclera and choroid, and a few were far enough anterior to elevate the pars plana. The thin sponges were fusiform on section, and the thick ones were lenticular.

The sclera was infiltrated with polymorphonuclear leukocytes in the early postoperative period, but the scleras of all the eyes examined eight weeks after operation were normal except in dog 24. The retina over the sponge was normal in all the animals except in sections which included the previously mentioned holes and disturbances of pigment. The retina over the sponge usually separated from the underlying choroid during the preparation of the sections; this indicated that there was not enough choroiditis to bind the two together. The choroid over the sponge was somewhat thinned and was less pigmented than undisturbed choroid, and it was intimately attached to the sponge, into which it had sent fibrous tissue, blood vessels, and pigment-bearing cells.

The histologic findings within the sponge spaces were those of a foreign-body reaction, with later replacement of inflammatory cells by fibrous tissue and blood vessels. At 24 hours after operation dense infiltration was seen in the interstices of the sponge by polymorphonuclear leukocytes (fig. 4). At five days these cells were less numerous, and fibrocytes could be seen invading the sponge from the choroid. Large cells were lined up on the surfaces of the sponge trabeculae. At seven days the cells on the sponge were well-developed multinucleated giant cells. By the 10th day the polymorphonuclear leukocytes had disappeared. The sponge was filled with fibroblasts, pink amorphous material, some mononuclear cells, and giant cells (fig. 5).

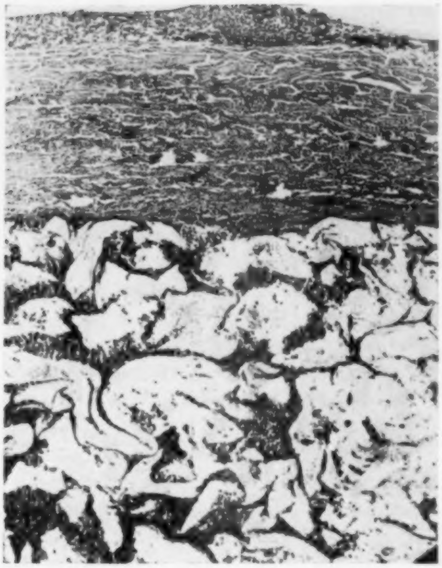

Fig. 4 (Kazdan, et al.). Dog 1. One day after operation. The sponge and the overlying sclera are infiltrated by many polymorphonuclear neutrophils. (Hematoxylin-eosin, reduced from ×90.)

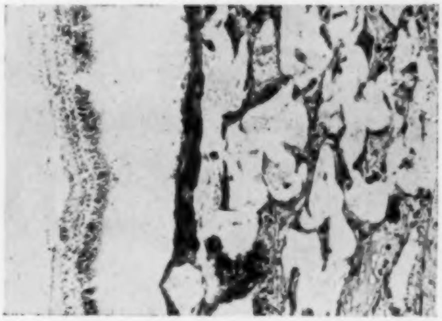

Fig. 5 (Kazdan, et al.). Dog 4. Ten days after operation. The polymorphonuclear neutrophils have disappeared, and the sponge interstices are now occupied by fibroblasts, foreign-body giant cells, macrophages, and a few inconspicuous blood vessels. (Hematoxylin-eosin, reduced from ×100.)

Four weeks after operation fibrous tissue was the predominant material in the sponge, and giant cells were decreasing in number. These persisted, however, until the 26th week. The next eye examined microscopically had been operated on 45 weeks before, and in it no sign of inflammation was present.

In all but one of the eyes examined more than 45 days after operation the picture was similar (figs. 6 and 7). The interstices in the sponge were filled with fibrous tissue, large, thin-walled blood vessels, and pigment-bearing cells. The last were most numerous in the inner half of the sponge. Some were multiprocessed melanophores containing finely divided melanin, and these were usually related to the blood vessels. There were also many histiocytes, which contained coarse clumps of pigment. That this was not blood pigment was demonstrated by a Prussian blue reaction for iron. The blood vessels consisted only of an endothelial wall and were most abundant toward the choroid and at the ends of the sponge.

The appearance of the sponge did not alter in any consistent manner with the passage of time. Some of the sponges looked somewhat fragmented, but others of the same age looked the same as those examined early. For

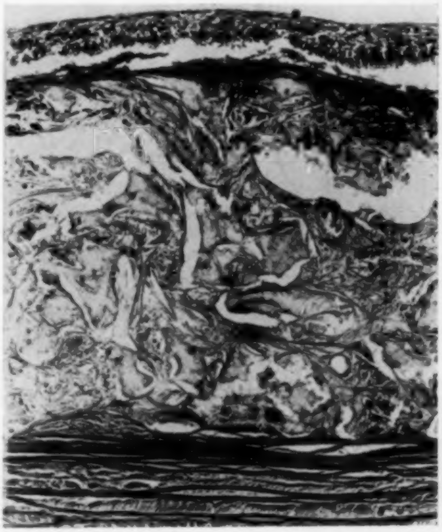

Fig. 6 (Kazdan, et al.). Dog 23. Fifty-six weeks after operation. The sclera and retina appear normal, and the choroid thinned. The sponge spaces are filled with fibrous tissue. (Hematoxylin-eosin, reduced from ×100.)

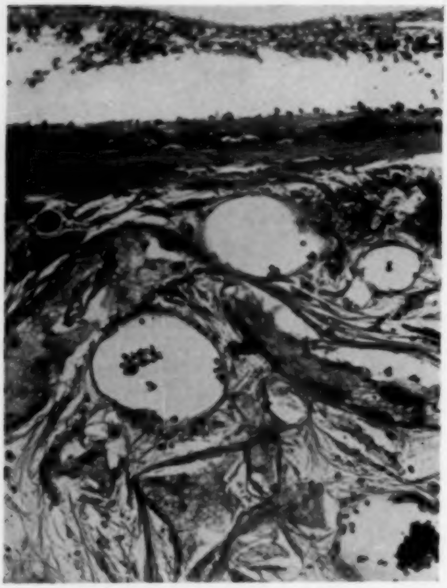

Fig. 7 (Kazdan, et al.). Dog 27. Fifty-nine weeks after operation. A high-power view of sponge near the ora serrata, showing the mature fibrous tissue and thin-walled blood vessels in the sponge typical in the long-term implants. (Hematoxylin-eosin, reduced from ×200.)

instance, the sponge from dog 23, examined at 56 weeks (fig. 6), looked about the same as that from dog 6, examined at four weeks.

Complications

Almost all the eyes withstood the operative procedure and the presence of the sponge well. In cases in which the eye was damaged by the operation or the sponge the disturbance was localized to the region of the implant and did not appear to be severe enough to have caused much loss of function of the eye as a whole.

The surgical manipulation was probably the cause of most of the damage to the retina and choroid; this certainly was the case in the four eyes from which vitreous was lost at operation. The surgeon had had no previous experience in ocular surgery, and as skill was developed, less trauma was inflicted on the eyes. In all the cases in which vitreous was lost operations were done early. There was enough reaction at the edges of the retinal holes to seal them down, and no retinal detachments resulted.

Choroidal separation due to hemorrhage was present in the eyes examined at one and 10 days after operation, but not in those examined after five and seven days. This suggests that hemorrhage into the suprachoroidal space was a common but not invariable complication. Possibly it occurred in other eyes as well, but the blood was absorbed by the time of enucleation.[17]

Two of the eyes were of special interest because of their unusual cellular reaction.

Vitreous was lost at the time of operation on dog 11, and the animal was killed eight weeks after operation. Examination revealed absence of the retina and choroid over about half of the sponge. The part of the sponge which was covered by choroid and retina had in it the fibrous tissue, blood vessels, and giant cells expected at this stage. The part which was exposed to the vitreous was filled with polymorphonuclear cells, and the ingrowth of fibrous tissue and blood vessels had been inhibited. In other eyes in which the sponge was exposed to the vitreous, such as that of dog 6, this inflammatory reaction was lacking. It is possible that it was present at one time and had disappeared by the time of examination, but the fact that the eyes looked quite usual suggests that a factor other than exposure of the sponge to vitreous was involved.

In the eye of dog 24, examined 56 weeks after operation, the reaction was more intense and more puzzling, because there was no apparent cause for it (fig. 8). The sponge was packed with polymorphonuclear neutrophils, which also infiltrated the overlying sclera. No blood vessels or fibrous tissue had grown into the sponge, in spite of the long period since operation. The choroid and ciliary body adjacent to the polyvinyl sponge were not involved by the inflammation, and the retina was normal. An infection would not be likely to produce such a well-localized

but acute-appearing reaction so long after the surgical procedure. An allergic reaction would probably have more eosinophils. Although the sclera was thinned and inflamed over the implant, it was not necrotic and even this eye, with the most severe reaction, could probably function well.

Descriptions of the individual eyes

The findings on examination of the eyes were as follows. As most of the eyes were normal externally save for the presence of scleral sutures, the gross appearance will be described only where there was an unusual feature. The time of examination, the type of operation, and the size of the sponge will be listed first.

Twenty-four hours after routine operation on dog 1. The sponge when inserted measured 11 by 5.0 by 3.0 mm.

Gross examination. The eye was cut sagittally. The sponge produced an elevation of the retina which extended from the ora serrata to 5.0 mm. behind it. The elevated retina was gray, and several fine folds were directed radially in it. A small hemorrhage was present in the base of one of these folds. There were a few small pink blebs or localized elevations of the retina over the implant. A slight wrinkling and pink discoloration in an area 1.0 mm. in diameter were present in the pars plana just anterior to the lateral scleral incision. The vitreous was clear, and the other ocular structures were normal.

Microscopic examination. The sponge in cross section appeared fusiform and extended posteriorly from the ora serrata. There was hemorrhage in the choroid and in the suprachoroidal space in all sections around the circumference of the choroid and extending anteriorly under the pars plana. In some sections there were small collections of blood between the sponge and the choroid, and in one subretinal fluid and hemorrhage were seen over the sponge. The retina was intact. The scleral incision was filled with polymorphonuclear leukocytes, and there was also a heavy infiltration of these cells between the scleral lamellae over the sponge (fig. 4).

The interstices of the sponge were filled with polymorphonuclear leukocytes and a few erythrocytes.

Five days after routine operation on dog 2. The sponge when inserted measured 11 by 5.0 by 3.5 mm.

Gross examination. The globe was sectioned horizontally. The retina and pars plana of the ciliary body were elevated over the sponge. Several vessels coursed over this part of the retina and appeared to arise from a puckering at the ora serrata. Several anteroposterior folds of retina and pars plana

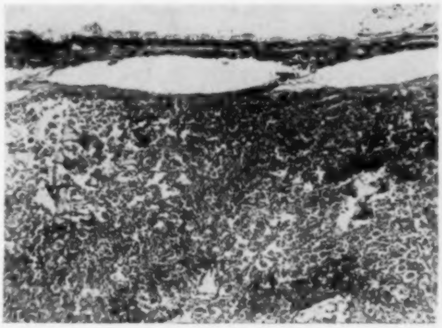

Fig. 8 (Kazdan, et al.). Dog 24. Fifty-six weeks after operation. The sponge is filled with polymorphonuclear neutrophils, and fibrous tissue and blood vessels are absent. The overlying pars plana is not affected by the inflammation. (Hematoxylin-eosin, reduced from ×100.)

were observed over the sponge, but there were no defects in the retina and no hemorrhage. The vitreous and other structures were normal.

Microscopic examination. The anterior end of the sponge was 1.0 mm. behind the ora serrata. The ends of the sponge were tapered. The choroid was intact and there was no choroidal or suprachoroidal hemorrhage or subretinal fluid or hemorrhage. The retina was normal. The infiltration of the sclera over the sponge with polymorphonuclear cells was less dense than in dog 1. The other structures were normal.

The inflammatory cells in the sponge were less numerous than in the eye examined after 24 hours. Most were polymorphonuclear, but some were mononuclear cells. There were a few erythrocytes, and an eosinophilic substance, which looked like fibrin, separated the cells. Large cells with abundant cytoplasm and several nuclei were lined up on the surfaces of the sponge. Blood vessels consisting of a single layer of endothelium invaded the sponge from the choroidal side.

Seven days after routine operation on dog 3. The sponge when inserted measured 11 by 6.0 by 3.5 mm.

Gross examination. On the surface of the globe there was a small mass of tissue which included the superior rectus muscle and the suture used to reattach it to the sclera. Otherwise, the eye was normal in shape. It was cut first horizontally, then vertically through the sponge. The sponge elevated both the pars plana and the retina, and extended 2.0 mm. posterior to the ora serrata. There was slight wrinkling of the retina along the ora serrata over the sponge, but there was no hemorrhage or retinal detachment. The vitreous and other structures were normal.

Microscopic examination. The sponge was fusiform on section and appeared compact. The sclera over it was infiltrated with polymorphonuclear cells

and mononuclear cells. In the choroid, pigment was being mobilized in macrophages. The retina was normal.

In the sponge the predominant cell was the polymorphonuclear leukocyte. Some free erythrocytes were present, and many giant cells covered the sponge. Fibrous tissue had begun to invade the anterior end of the sponge, and blood vessels were growing in from the choroid. A few pigment-bearing macrophages were present.

Ten days after routine operation on dog 4. The sponge when inserted measured 15.5 by 4.0 by 1.5 mm.

Gross examination. The sponge caused a distinct elevation of the posterior part of the pars plana and the anterior part of the retina. On the medial end of the elevation was a circular pink area 2.0 mm. in diameter, which on section proved to be a slight elevation of the retina by subretinal fluid and a few erythrocytes. The retina was otherwise normal. The vitreous was clear, and other structures were normal.

Microscopic examination. The sponge underlay both the retina and the posterior part of the pars plana. It was 3.0 mm. anteroposteriorly and 0.5 mm. thick. A suprachoroidal hemorrhage, about the thickness of the retina, entirely separated the choroid in each section. The choroid, however, appeared intact, and the retina was normal.

At this stage there were no polymorphonuclear leukocytes, and only a few lymphocytes in the sponge. The interstices of the sponge were filled with fibrin, more fibroblasts than found in the sponge of dog 3, and a few multinucleated giant cells. There were a few thin-walled blood vessels in the sponge, but they were not conspicuous. Pigment-bearing macrophages were scattered in the sponge, particularly near the choroid (fig. 5).

Twelve days after operation on dog 5. The sponge was introduced into the right eye by pushing it into the suprachoroidal space through one scleral incision. When inserted it measured 15 by 5.0 by 3.5 mm.

Gross examination. The eye appeared normal except for the scar of the paracentesis and the scar in the sclera. The globe was sectioned obliquely to the nasal side of the sponge. The sponge had elevated the retina about 2.0 mm. This elevation which straddled the ora serrata measured 5.0 mm. anteroposteriorly and 3.0 mm. in width. There was no retinal detachment, and the retina appeared normal except for some pigment mottling most marked on the side of the eye opposite the sponge. This mottling was also present in the other eye. The vitreous was clear, and the other structures were normal. Microscopic sections were not made.

Twenty-three days after operation on dog 6. At operation scleral incisions were made at the positions of 9-, 12-, and 3-o'clock. The end of the long strip of sponge was pulled through the suprachoroidal space from 9- to 12-o'clock, and then from 12- to 3-o'clock. A considerable amount of vitreous was lost from the nasal, or 9-o'clock, incision. The sponge when inserted was 23 by 3.0 by 1.5 mm.

Gross examination. The eye was opened in the coronal plane behind the sponge, which was just posterior to the ora serrata. The sponge produced slight elevation of the retina. Over the nasal part of the sponge, where vitreous had been lost at operation, there was a ragged retinal tear, but the retina was not separated from the underlying choroid. The vitreous was clear, and the other structures were normal.

Microscopic examination. The sponge extended around a third of the circumference of the globe. It was 22-mm. long measured on the arc, and 0.7-mm. thick where covered by the choroid, and 1.3-mm. thick where the choroid was defective. The defect in choroid and retina was 3.3-mm. long in the coronal plane. The ends of the sponge failed to reach the nasal incision by 1.8 mm., indicating that it had retracted. The sclera was normal except for the incisions, which were filled with cells. There was relatively little reaction to the silk sutures. Where the choroid was present, it appeared normal. The defect in the retina was the same size as that in the choroid. In some areas of the retina over the implant there were hyperplasia of the pigmented epithelium and some migration of pigment into the retina. Toward the edge of the break the retina looked atrophic, but for the most part it appeared normal.

Fibrous tissue had invaded the sponge from both the sclera and the choroid. The predominating cell in the interstices of the sponge was the giant cell. These were lined up on the surfaces of the trabeculae, and some of them contained clumps of pigment. A few lymphocytes and macrophages were laden with clumps of melanin, but polymorphonuclear leukocytes were absent.

New blood vessels invaded the sponge, but appeared to be less numerous in the part of the sponge exposed to the vitreous than in the part covered by choroid and retina. In some sections there were small recent-appearing hemorrhages between the choroid and the sponge, and preretinally. Unlike all the other cases, this eye was enucleated from the living animal, and the optic nerve was clamped before it was cut. Perhaps the preretinal hemorrhage, which was the only one seen in the series, resulted from a sudden regurgitation of blood into the retinal vessels on clamping the optic nerve.

Four weeks after routine operation on dog 7. The sponge when inserted measured 8.0 by 3.0 by 1.5 mm.

Gross examination. The eye was capped horizontally. The implant had not elevated the retina visibly. Over the implanted sponge there were a few fine transverse retinal folds. The retina was elsewhere in normal apposition to the choroid, and it was intact. The vitreous and other structures were normal.

Microscopic examination. The sponge was fusiform on section and about 0.5-mm. thick. The sclera and choroid were normal, and the retina was normal but detached by artifact.

The interstices of the sponge contained both fibrous tissue and giant cells. Blood vessels were present, but were not prominent. Pigment-bearing macrophages were scattered throughout the sponge.

Five weeks after operation on dog 8. The operation was done to demonstrate that it is possible to elevate the choroid of most of the arc of two quadrants of the eye by routine method, using only two scleral incisions for the introduction of the sponge. The two incisions were 23 mm. apart, just superior to the lateral and medial recti, and started 10 mm. behind the limbus. The operation was accomplished ....without complication. The sponge was 23 by 5.0 by 3.0 mm.

Gross examination. The eye was capped in the frontal plane just behind the position of the sponge. As it was being cut, the retina could be seen to be peeling off in the superior half of the eye. The sponge produced an elevation of the choroid and of the retina, which fell back against the choroid. These structures were otherwise normal. The vitreous was clear, and the other ocular structures were normal.

Microscopic examination. The sponge which was cut lengthwise was 1.5 mm. thick. Some of the sections showed hemorrhage in the suprachoroidal space, which was widened to the thickness of the sclera for a short distance. Other sections did not show hemorrhage.

The picture within the sponge was similar to that seen at four weeks. The sponge was intimately attached to both the sclera and the choroid (fig. 3) and its interstices were filled with fibrous tissue, macrophages, blood vessels, and giant cells.

Five weeks after routine operation on dog 9. The sponge was compressed from an original thickness of 10 to 3.0 mm. while being boiled. The dimensions of the sponge prior to insertion in the eye were 20 by 5.0 by 3.0 mm. The sponge was inserted in the routine fashion, without complications.

Gross examination. The eye was normal in size and shape before being opened. It was cut in the sagittal plane, through the sponge, and the retina was torn in the process. Examination of the opened eye showed that the sclera was bulged slightly outward by the sponge, but appeared healthy. The choroid and retina were displaced inward by the sponge and most of the space used for the sponge was the result of this displacement. Except for the tear in the retina, most, and perhaps all, of which was made at the time the eye was capped, the ocular structures were normal.

Microscopic examination. The sponge was 2.3-mm. thick, and, although its ends were rounded, it was not shaped by the tissue pressures as much as were the other sponges. The sclera was closely attached to it and tended to follow its contour. The sclera was normal in thickness and was not infiltrated by cells. The choroid also was closely attached to the sponge in most places, but near the center of the sponge there were a couple of small folds in the choroid. The retina was not attached to the surface of the bulge in the sections, but it was in the gross specimen, except for part, which was torn. The edges of the retinal breaks did not show healing.

The interstices of the sponge were reduced only slightly in size by the compression. They were filled with fibrous tissue. Only a few giant cells were present, and blood vessels were present, but only in small numbers in the sponge. Pigment-bearing macrophages were scattered around the outer parts of the sponge, especially near the choroid.

Seven weeks after operation on dog 10. The sponge was placed in the posterior part of the globe. Radial incisions were made superiorly 8.0 mm. apart with their anterior ends 13 mm. behind the limbus. The sponge was also wider (larger anteroposteriorly) than most other implants. Its dimensions before insertion were 8.0 by 6.0 by 3.5 mm. The operation was accomplished without complication.

Gross examination. The posterior end of the medial incision was about 6.5 mm. above the optic nerve. A small mass of fibrous tissue was present on the surface of the sclera to the temporal side of the superior rectus muscle, between the incisions and extending to 11.5 mm. from the limbus at the 2-o'clock position. The eye was opened in the frontal plane at the equator. The elevation produced by the sponge was superotemporal to the disc, and its posterior margin was 6.0 mm. away from it. The elevation was 7.0 mm. in the transverse direction, 4.0 mm. anteroposteriorly, and about 1.5 mm. high. At the temporal end of the elevation there was a small hole in the retina, about 1.0 mm. in diameter. The retina was not detached, and the vitreous was clear.

Microscopic sections were not made, as the eye was saved for demonstration.

Eight weeks after routine operation on dog 11. A piece of thin sponge measuring 16 by 3.0 by 1.5 mm. was implanted with the usual technique. As the blade of the iris repositor was being passed between the sclera and choroid, it met an obstruction. It was finally forced through so that its tip emerged from the second incision. The choroid was folded over the tip and also emerged from the incision, but with a little manipulation, it slipped back into place. No vitreous was lost.

Gross examination. The scleral wounds were well healed. The eye was capped sagittally. The slight elevation produced by the implanted sponge medial end, the retina and choroid over the sponge extended 2.5 mm. behind the ora serrata. At its were thin and the pigment was disturbed. The sclera also appeared thinned over the sponge, and the medial suture had gone through the sponge. The vitreous and other structures were normal.

Microscopic examination. The sponge was fusiform and was 1.3-mm. thick. Over it, the sclera bulged outward and was thinned. The retina and

choroid were intact and normal in appearance over part of the sponge, but were absent over it in other slides. Histologically the sponge was strikingly different in these two areas. In the part of the sponge covered by retina and choroid, the spaces were filled with connective tissue. There were only a few giant cells and no polymorphonuclear cells. A few histiocytes were present, and vascularization was well advanced. This is the picture one would expect to see at this stage. The sclera in these sections was normal in thickness and was not inflamed.

In the sections in which the retina was defective over the sponge, the choroid was thinned and was represented by strands of connective tissue and clumps of pigment-bearing cells. The sponge was densely infiltrated by polymorphonuclear leukocytes. There were few giant cells, and fibrous tissue and blood vessels were absent. The overlying sclera was thinned and infiltrated by polymorphonuclear leukocytes.

Eight weeks after routine operation on dog 12. A thick sponge measuring 10.3 by 7.0 by 4.5 mm. was inserted by the usual method. There was a considerable amount of hemorrhage at operation, and vitreous was lost from the lateral incision.

Gross examination. The eye was normal in shape and the scleral wounds were well healed. The eye was cut sagittally. The elevation due to the sponge was apparent and began at the posterior part of the pars plana. The retina was not detached any place and appeared normal except for an area 2.0 mm. in diameter just lateral to the lateral end of the sponge, where depigmentation and clumping of pigment were evident. The vitreous was clear.

Microscopic examination. The sponge was fusiform in outline, measured 5.0 mm. anteroposteriorly, and was 1.5-mm. thick. The sclera was normal and was closely applied to the sponge into which it could be seen sending strands of fibrous tissue. The choroid was intact over the sponge. The retina in the sections was normal, but was separated from the posterior part of the sponge. This was probably due to artifact because there was no subretinal fluid, and this separation was not noted at gross examination.

The picture within the sponge was similar to that seen at five weeks. Most of the space was taken up by fibrous tissue, but there were some large blood vessels and some macrophages containing pigment. A few giant cells were still to be found, and they seemed to be most numerous near the choroid. A vessel could be traced from the choroid into the sponge.

Ten weeks after routine operation on dog 13. Cautery was used to stop scleral bleeding originating behind the insertion of the superior rectus muscle. The sponge used measured 8.0 by 3.0 by 1.5 mm.

Gross examination. The wounds were well healed. The eye was opened in the sagittal plane. The sponge elevated the posterior part of the pars plana and the anterior part of the retina. Near the lateral end of the sponge there was a retinal hole less than 1.0 mm. in diameter. Near it was a small, oval, yellow area. At the medial border of the sponge an area of disturbed pigmentation measured 2.0 mm. in diameter. The retina was not detached. The rest of the retina and the vitreous were normal.

Microscopic examination. The sections were made through each of the three areas of retinal disturbance and through areas where the retina and choroid overlying the sponge were normal. The retina gradually thinned toward the margin of the hole, and at its margin there were large clumps of pigment in the nerve-fiber layer. At the margins of the hole the retina became attached to the underlying tissue, which was fibrous tissue replacing the choroid in that area.

The yellow area referred to above was an area of retinal thinning. The retina was half its normal thickness. The layer of rods and cones was absent, and the nuclear layers were thinned greatly. There was slight thinning of the nerve-fiber layer.

In the area showing disturbance of pigment, pigment-bearing macrophages were scattered in the retinal layers, and the retina was thinned in places.

The sclera was normal in some sections, and in others contained pigment-bearing macrophages between its fibers. The spaces within the sponge were occupied by fibrous tissue, thin-walled blood vessels, giant cells applied to the trabeculae of the sponge, scattered pigment, and macrophages containing pigment. Some of the spaces in the sponge were filled with an amorphous red material, possibly fluid.

Twelve weeks after routine operation on dog 14. A thin but wide sponge was inserted to prove that a large area of choroid can be elevated by sponge. Its dimensions were 8.0 by 8.0 by 1.5 mm. There were no complications at operation, and no vitreous was lost.

Gross examination. The shape of the eye was normal. The eye was cut open in the horizontal plane. No elevation of the retina was visible, and no retinal lesions were seen. The vitreous was condensed behind the lens, and white strands of vitreous were lying on the retina. Most of the vitreous was clear.

No *microscopic sections* were made, as the eye was saved for demonstration.

Thirteen weeks after routine operation on dog 15. The sponge measured 15 by 3.0 by 1.5 mm.

Gross examination. The eye was normal in shape. It was cut open in the horizontal plane. The sponge was too thin to produce a visible elevation of the retina. The retina was grossly normal. Most of the vitreous was clear and gelatinous, but there was a firmer mass of it in the superior temporal quadrant of the eye, behind and adherent to the temporal third of the lens, extending back to the equator, and giving off strands which went back to the retina just above the disc. This mass was translucent and perhaps resulted from fixation of the eye. The other ocular structures were normal.

Microscopic examination. The superior half of the eye was sectioned sagittally through the sponge. The sponge measured 0.6 mm. in thickness and 2.8 mm. anteroposteriorly. In two slides in which eosinophilic vitreous was seen, there was localized thinning of the retina for about 1.5 mm. at the equator. All the retinal layers were thinned, and part of the layer of rods and cones of the outer nuclear layer was lost. Macrophages containing pigment were present in the outer layers of the atrophied area of retina. In other sections the retina and the choroid, including that over the sponge, were normal. The sclera was normal.

In the sponge there were fewer giant cells than were seen in eyes examined sooner after operation. The spaces were filled with fibrous tissue, pigment-containing cells, and thin-walled blood vessels.

Twenty-six weeks after operation on dog 16. Three scleral incisions were used for the introduction of the sponge, as in dog 6, and in this animal also vitreous was lost from the nasal incision. The sponge measured 19 by 3.0 by 1.5 mm.

Gross examination. A 2-mm. thick mass was present on the surface of the sclera between the 9:30- and 2:30-o'clock positions from the limbus to the equator. The eye was cut sagittally. The sponge was partly under the ciliary body and partly under the retina. The retina was in normal position and was generally normal, but had the following lesions:

1. Near the ora serrata superiorly was a 0.5-mm. spot of pigment.
2. Slightly temporal to this was a slight localized elevation of the retina and adjacent pars plana; the latter was lighter than normal.
3. Posterior to this were three white spots in the retina measuring from 0.5 to 2.0 mm. in length.
4. Nasal to the sponge, at the 9-o'clock position, there was a 0.5-mm. hole in the retina, surrounded by a narrow zone of increased pigmentation.

The vitreous was clear, and the other structures were normal.

The sponge was 2.2 mm. anteroposteriorly and 1.0 mm. thick. The sponge material appeared to be broken up into smaller spicules than in previous eyes. The interstices were filled with fibrous tissue, which was moderately cellular, some blood vessels, and pigment-bearing cells. A few giant cells were still present at this stage.

Twenty-six weeks after routine operation on dog 17. The sponge was about 18 by 3.0 by 1.5 mm.

Gross examination. The eye was normal in shape. It was cut before fixation so that the interior could be examined in the fresh state. As a consequence, the fixed eye was misshapen. The sponge produced no visible elevation. The retina and other structures appeared normal except for a 1.5-mm. thinning of pigment, shown by transillumination, under the anterior temporal suture. The vitreous was clear. No sections were made.

Forty-three weeks after routine operation on dog 18. The sponge was 9.0 by 5.0 by 3.0 mm.

Gross examination. The implant produced a slight elevation of the choroid and retina. The retina was normal except for a 1.0-mm. hole at the ora serrata 2.0 mm. temporal to the temporal suture. A strand of vitreous was attached to the edge of the hole. The retina was not detached. The other ocular structures were normal.

Microscopic sections were not made and the eye was saved for demonstration.

Microscopic appearance in eight of the nine eyes examined 45 to 59 weeks after operation.

The microscopic appearance of the sponge was essentially the same in eight of these nine animals (dogs 19 to 26) which were studied this length of time after operation. The sponges were found to be tapered as previously noted. They did not appear different from those eyes examined soon after operation. The interstices were filled with mature fibrous tissue, and endothelium-lined blood channels were prominent. There were occasional small collections of mononuclear cells, but multinucleated giant cells, as seen earlier, and polymorphonuclear cells, as seen initially, were absent. The blood vessels were most numerous near the choroid and toward the ends of the sponges. Melanophores appeared to be related to these vessels and were almost confined to the inner third of the sponge. Round macrophages containing large clumps of melanin were scattered in the inner portion of the sponge. A Prussian blue stain of sections from dog 23 showed no blood pigment in these cells. In general, in these cases, the sclera was normal, and for the most part the retina and choroid were normal, although small areas of damage to these structures were found frequently. The retina was not detached in any case.

Forty-five weeks after operation on dog 19. The sponge was pushed into the suprachoroidal space through a single incision parallel to the limbus. The sponge measured 8.0 by 4.5 by 3.5 mm.

It produced an elevation of 1.5 mm. The retina was intact, but the sclera over it was slightly thinned.

Forty-eight weeks after routine operation on dog 20. The sponge was thick, measuring 7.5 by 7.0 by 4.0 mm.

The choroid and retina were intact except for a 0.5-mm. area of loss of pigment under one nasal suture. Sponge was 4.2 mm. anteroposteriorly and 1.3 mm. thick.

Fifty weeks after routine operation on dog 21. The sponge was thick and wide, 9.5 by 10 by 8.0 mm.

A small amount of vitreous was withdrawn by a needle. A thin mass of tissue was adherent to the superoanterior portion of the globe. One hole in the retina and choroid was at the site of the needle puncture, and a vitreous band was attached to it. A second hole was under the nasal incision in the sclera. A large, lens-shaped elevation of the choroid

and retina was produced by the sponge (fig. 2). The sponge was 6.9 mm. anteroposteriorly, and 4.4 mm. thick.

Fifty-five weeks after operation on dog 22. The sponge was inserted between two incisions parallel to, and 9.0 and 14 mm. behind, the limbus in the superonasal quadrant. The sponge was 5.0 by 3.0 by 1.5 mm.

Two small holes were found in the retina and choroid each of which was under a scleral suture. A fine vitreous band was attached to each hole, but the retina was not separated from the choroid. The sponge produced a slight elevation.

Fifty-six weeks after routine operation on dog 23. Paracentesis was not performed and one application of diathermy was done for scleral bleeding. Sponge measured 5.0 by 3.0 by 1.5 mm.

The retina was absent over much of the sponge, and its torn edge was adherent to the underlying choroid. The choroid over the implant was thinned and had a less than normal amount of pigment (fig. 6). In some sections the choroid and retina over the sponge were normal.

Inflammatory cells in the sponge at 56 weeks after operation on dog 24. The operation was done on the left eye by the usual method, and there were no complications at that time. At its close, antibiotic-containing ointment (Neosporin) was instilled in the conjunctival sac. This was done also in dogs 25 and 26, neither of which had unusual cellular reactions. The sponge inserted measured 11 by 3.0 by 1.5 mm.

Examination. The bulbar conjunctiva of the left eye was red while the animal was living, but there was no chemosis or discharge. The cornea and iris were normal, and the media were clear. The right eye was normal.

The enucleated left eye was normal in appearance except for a brown rounded mass of tissue between the 12- and 1:30-o'clock positions which started 3.5 mm. behind the limbus. The mass was 5.0 mm. in diameter and was elevated about 3.0 mm. above the contour of the globe. One silk suture was present 10.5 mm. behind the limbus at about the 3-o'clock position. The mass of tissue was similar to those seen in dogs 3, 16, and 21, except for being darker and thicker.

The eye was cut open from the 12- to 9-o'clock positions, in an anteroposterior plane. The elevation was too slight to be seen before a section was made through the sponge. Beneath the external swelling the choroid was reddish and looked thin in an area 2.0 by 3.0 mm. Under the temporal suture was an area which looked like healed choroiditis. It was less than 1.0 mm. in diameter. The vitreous was clear.

Microscopic examination. The picture was unlike that in any other eye studied and bore a closer resemblance to the eye examined 24 hours after operation than to any other. The sponge was in the suprachoroidal space and had the usual tapering of its edges. It was 1.0 mm. thick, which was not unusual for sponges which had been 1.5 mm. thick at the time they were inserted.

The sponge was packed with polymorphonuclear leukocytes and many macrophages containing blood pigment, as shown by iron stain (fig. 8). Free blood was present in the posterior part of the sponge, but there were no blood vessels. Fibrous tissue was absent, and foreign body giant cells were present only in small numbers. Small numbers of mononuclear cells were scattered among the polymorphonuclear cells.

The pars plana of the ciliary body and the choroid, both of which were in contact with the sponge, were unaffected by the inflammation. The overlying retina also was normal. The sclera next to the sponge was densely infiltrated by the acute inflammatory cells and was somewhat thinned. The mass on its surface consisted of fibrous tissue densely infiltrated by polymorphonuclear leukocytes.

Fifty-seven weeks after routine operation on dog 25. The sponge measured 10.5 by 3.0 by 1.5 mm.

The sponge did not produce a grossly visible elevation. The retina and other structures were normal.

Fifty-seven weeks after routine operation on dog 26. Vitreous was lost through the nasal incision. The sponge measured 7.0 by 3.0 by 1.5 mm.

Examination. The sponge did not produce a grossly visible elevation. There was a hole in the retina and choroid at a point corresponding to the posterior part of the nasal scleral incision, from which the vitreous was lost at operation. A thin white band of vitreous passed from the hole to the posterior surface of the lens. There was another small retinal hole under the temporal incision, but the vitreous here was normal. The retina was otherwise normal.

Fifty-nine weeks after routine operation on dog 27. The spatula pushed the choroid ahead of it as it emerged from the nasal incision, but no vitreous was lost. The sponge was 12 by 3.0 by 1.5 mm.

Examination. A hole in the retina and choroid, 2.5 mm. in diameter, exposed the central part of the sponge to view from the interior of the eye. The margins of the defect were bound down to the sponge, and the retina was not detached. The vitreous was normal.

Comment

Control over the position of the sponge seemed better when it was drawn between two scleral incisions than when it was thrust into the suprachoroidal space through one incision. It was easier to incise the sclera when the eye was firm than when it was soft, so that in clinical use, subretinal fluid should

not be released until after the scleral incisions have been made. In a few instances we encountered resistance when passing the spatula between the choroid and the sclera, and the choroid in these cases was found to have been damaged. The trauma of the operation is the most important hazard of the implantation of polyvinyl sponge, for the reaction to the material itself was usually harmless to the eye. The small areas of retinal and choroidal damage which were found in some of the eyes did not appear to be of great consequence.

The sponge in the eye was always thinner than it had been prior to its insertion. The decrease in thickness was present in the cases studied soon after operation and in those studied later, and was caused by the compressing effect of the tissues rather than by absorption of the sponge. This fact is demonstrated by the eye of dog 6, in which there was a hole in the retina and choroid over part of the sponge. That part of the sponge was almost twice the thickness of the part covered and compressed by the choroid and retina.

In its clinical application to the treatment of retinal detachment, this procedure could be combined with diathermy to close retinal tears and bind the retina to the choroid. Implantation of vitreous would push the retina toward the elevated choroid and would help to hold it in place. Paracentesis should not be required in most cases, as the release of subretinal fluid should make the eye soft enough to receive the sponge without a dangerous rise of the intraocular pressure. The choroid could be elevated for part or for all of the circumference of the globe; the anteroposterior dimension of the elevation and its height also could be selected to fit the requirements of the individual patient. A marked elevation of the choroid, such as the 3.5-mm. elevation in the eye of dog 21, should be comparable to the inward displacement of the choroid obtained by scleral buckling. A broad but thinner sponge, such as the one, 8.0 by 6.0 by 3.5 mm., used in dog 10 would have an effect similar to scleral shortening. It would be of value in cases in which the retina is prevented from returning to its normal position by fixed folds or vitreous adhesions.

The permanence of the choroidal elevation produced by the polyvinyl sponge would permit sufficient time for the diathermy reaction to bind the retina to the choroid, and it also would prevent traction on the retina such as would occur if the choroid returned to its original position. This feature would be important when the retina is weakened by senile degeneration, myopia, or previous operation.

In most of the microscopic sections some separation of the retina had occurred over the elevated choroid, although the retina was usually normal in appearance and there was no subretinal fluid. This artifact demonstrates that sufficient inflammation did not occur to bind the retina to the choroid.

Summary

Techniques for the insertion of polyvinyl sponge into the suprachoroidal space were described. In one method two parallel incisions were made through the sclera, and the choroid between them was separated by sweeping motions of a spatula, as the ciliary body may be separated from the sclera in cyclodialysis. An iris repositor with a hole drilled through the tip of its blade was passed into the suprachoroidal space from one incision to the other and a strip of sponge was tied to its emerging tip. The blade was then drawn back out of the suprachoroidal space, pulling the sponge into position in the eye. The sponge was cut flush with the sclera, its ends were tucked into the suprachoroidal space, and the scleral incisions were sutured.

Another technique was to make one incision in the sclera parallel to the limbus, separate the choroid posterior to it with a cyclodialysis spatula, and push the sponge into the space thus prepared for it.

The operation was performed on one eye of each of 27 dogs. Ophthalmoscopically, there was little disturbance of the eyes. There

was vitreous hemorrhage in one dog, but absorption had occurred by the time of enucleation at 50 weeks. One choroidal separation was observed postoperatively, but it was not present at microscopic examination eight weeks after operation. Most of the sponges were inserted near the ora serrata, and elevation of the retina in that region was difficult to see with the ophthalmoscope.

Gross examination on all 27 eyes revealed that the operation and the presence of the sponge were tolerated well. In 11 eyes there were small holes in the retina and choroid over or near the sponge, and in an additional four eyes there were small areas of retinal and choroidal pigment disturbance in the region of the sponge but no holes. The vitreous was usually normal. In four eyes slender bands of vitreous were present and took origin at a retinal hole. The retina was not detached in any of the eyes.

Microscopic examination of 21 eyes revealed that the cellular reaction to the sponge consisted at first of an acute inflammation in the sponge itself and in the overlying sclera, then of the appearance of foreign body giant cells, fibrous tissue, and blood vessels in the sponge, and, finally, of the occupation of the sponge by mature-appearing fibrous tissue with no reaction in the surrounding ocular tissue. There were two exceptions to this picture. In one case acute inflammatory cells filled the sponge and infiltrated the overlying sclera, but did not involve the choroid or retina, 56 weeks after operation. Most of the eyes, however, were very tolerant of the sponge, which was incorporated into the tissues of the eye. It is interesting that although the polyvinyl sponge was near the incisions in the sclera, it was not extruded in any case.

Elevation of the choroid and retina (1.3 mm. or more) was present in the seven eyes which contained sponge 3.5 mm. or more in thickness at the time of operation. The one precompressed sponge which was used was particularly effective in elevating the choroid; it was 3.0 mm. thick at the time of insertion and 2.3 mm. thick in the eye. An uncompressed sponge, 8.0 mm. thick, was inserted and it produced an elevation of the choroid and retina of 3.5 mm. This study demonstrated that even the posterior part of the globe is accessible for the insertion of sponge. Strips of sponge as long as 23 mm. were inserted into the suprachoroidal space. The elevation produced by the polyvinyl sponge was present even at 59 weeks after operation, and there was no indication that it had changed with the passage of time.

This procedure may be applicable to the surgical treatment of retinal detachment in man.

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Discussion

Dr. William G. Everett (Pittsburgh): The surgical treatment of retinal detachment has gone through several stages with which we are all familiar. The first rational treatment for retinal detachment was outlined by Gonin. We are now in the midst of the second stage, that of alteration of the globe to reduce the vitreous cavity or to inwardly displace the choroid.

We all await the next stage which will, we hope, give us some idea as to the basic pathologic factors which give rise to retinal detachment. The clinical work of Shaffer and the laboratory studies of Wadsworth and Baláz on the role of the vitreous in retinal detachment suggest that we may be entering the third stage of development and will possibly find a more satisfactory approach to this frustrating problem.

The paper presented today further extends our knowledge of the second stage of treatment of detached retina as another means of inwardly displacing the choroid. It differs in that it employs the subchoroidal space to obtain this displacement.

As the authors have mentioned, Strompelli reported on the placing of gelatin sponge in the suprachoroidal space in 1954. Earlier, in 1933, he used plasma for a similar purpose. Dellaporta reported the use of citrated whole blood in a similar fashion in 1955.

The obvious advantage of the authors' method is the permanency of the choroidal displacement, as borne out by their paper today.

Of the multitude of procedures conceived for alteration of the globe, few have been studied first in the animal before applying them to detachments in humans. I think the authors should be commended for their careful analytic laboratory studies before making clinical application of their operation.

I believe a word of caution is warranted in interpreting the findings in animal studies. There are decided differences between animal eyes and human eyes with retinal detachment. The well-formed vitreous body of the animal and unusual resistance of animals to retinal detachment is most important. The same firm vitreous structure creates undue pressure in the area of the operation in all animal experimental scleral shortening or vitreous volume reducing procedures, thus altering the normal manner of healing of the globe.

The vascularity of tissues in animal eyes varies from that in humans. This is especially true of the sclera of the dog, which is most vascular.

Most important of all, the difficulty of producing a detachment in an animal must be remembered. The fact that detachment of the retina does not occur in an animal following an experimental eye operation is of little or no significance as regards the application of this operation to the treatment of retinal detachment in humans.

The principles of the authors' approach seem to me to be quite feasible, and it would seem that it is possible to treat some retinal detachments in humans by this method.

The absence of inhibition of the fibroblastic infiltration of polyvinyl sponge in areas exposed to direct contact with the vitreous is most interesting and should be studied further. It might be of interest to study the vitreous cells in relationship to this area to see if they contribute to this inhibition.

The major question in my mind regarding the clinical applicability of the procedure is the incidence of hemorrhage which may result from placing the sponge. The spatulization of the suprachoroidal space and the trauma of inserting the sponge seem to be real dangers. In my original description of scleral outfolding, I spatulized the suprachoroidal space in a similar fashion but, after a short period of clinical trial, I found that even the most careful spatulization of the suprachoroidal space caused occasional hemorrhage; that is why

I discontinued this step of the operation.

This step is essential to the procedure discussed by the authors, and perhaps they will be able to give us more detailed clinical experience which will answer the problem of whether or not choroidal hemorrhages are too frequent.

So frequently in operating on detachments of the retina we find ourselves working in the superior temporal quadrant in intimate association and relationship to the superior vortex vein. Here I think one would have to use extreme care and judgment in placing the sponge, because of the intrascleral course of the vortex vein and our lack of knowledge of its exact location as it joins with the choroid.

I also wonder what modifications may be necessary in the procedure as regards the application of diathermy. Ideally, the polyvinyl sponge should be placed over the retinal tear, it would seem, to override the detached area, with the tear in the center. One also wants diathermy in this same area. I wonder if the diathermy would be given through the sponge or placed in the area of the tear prior to the placement of the gelatin sponge. Inserting a sponge through a previously diathermized area might prove hazardous. On the other hand, the control of diathermy current passing through the sponge might be difficult.

Dr. P. Robb McDonald (Philadelphia): I have not read this paper, and undoubtedly there are certain points in it that would obviate or perhaps answer some of the questions that Dr. Everett had. I have exactly the same questions.

I would think that insertion of a sponge underneath the sclera, especially in previously operated-on eyes, might lead to dangerous loss of vitreous. I used to introduce a spatula under the sclera before doing a full thickness resection, but I don't do full thickness resections any more. In this procedure if you are going to apply diathermy, you have to apply the diathermy to the sclera first, and after that probably make the scleral section and put the sponge underneath.

I believe the chances of loss of vitreous in the clinical case would be quite great, as well as the chances of hemorrhage. It is a very interesting approach, however, and it is only by investigative work like this that we finally will come to some answer as to how we can push the choroid inward to meet the retina in those cases in which we cannot get the retina back to the choroid.

Dr. John W. Henderson (closing): Although this experimental procedure certainly seems to have some application in retinal detachment repair, still I do not want anyone to have the idea that we have invented another operation. This work was undertaken first to investigate further the tolerance of the eye to implantations of foreign material within its substance.

Secondly, there was the question of whether it would have any clinical application in the repair of retinal detachments, for when the procedure was conceived several years ago ophthalmologists were at the climax of inventing methods to better repair retinal detachments by reducing either the volume or the circumference of the globe.

The practicality of any experimental work is whether it can be applied to the clinical subject. We have done so with this polyvinyl sponge operation. Over a year ago we placed the sponge in a woman's eye who had already lost the vision of one eye as a result of retinal detachment, and whose remaining eye possessed a detachment that extended over three-fourths of the circumference of the globe. We identified at least 10 holes in the retina.

The initial operation on the eye that I wish to tell you about consisted of penetrating diathermy pins across the area corresponding to the holes as near as we could approximate the pins to the holes. This procedure was supplemented by a scleral fold as well as implantation of 1.0 cc. of vitreous; then finished off with surface diathermy. This brought about no settling of the retina at all.

One week later we placed several more penetrating pins in an attempt to try to ring off the holes that we felt we might have missed at the first operation. We instituted the usual subretinal drainage and injected 1.5 cc. of air. We added no more vitreous at that time.

The second operation brought about a reattachment of the retina, which lasted for about three weeks, and then the retina came loose again. Nearly a month after the initial operation and about three weeks after the second operation we put in the sponge material as described by Dr. Kazdan. The sponge we used was 8.0-mm. long, 5.0-mm. wide, and 2.0-mm. thick. The operation was supplemented by an injection of 2.0 cc. of air.

Of course it would be nice to be able to say that this was the operation that worked but it didn't work any better than the others. Nevertheless, we have followed this patient for over a year and have found that the sponge did not extrude through the sclera. Neither has it intruded into the vitreous cavity. The patient has no complicated cataract, and vision is still limited to hand movements. I think it is interesting that in this individual for a period of a year the eye has been very tolerant of the material that was inserted.

I agree with both the discussers, who have had a great deal of clinical experience in working around the eye, that choroidal hemorrhage is to be reckoned with in any operation designed to insert anything in the suprachoroidal space. Without having had more experience with this sponge, I would say that I probably would not apply diathermy to the globe in the area in which the sponge is inserted. The reaction over the sponge may be sufficient to replace the effect of diathermy.

OBSERVATIONS ON ACCOMMODATIVE CONVERGENCE*

Especially its nonlinear relationships

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Under normal conditions, a stimulus to change the accommodation of the eye is accompanied by a change in the stimulus of convergence, which will be manifest as a change in the phoria of the eyes. The magnitude of the change of convergence (phoria) in prism diopters caused by a given change in the accommodation (diopters) is called the "accommodative convergence:accommodation ratio" (AC:A) and is usually expressed in prism diopters per diopter. This ratio can be found in several ways. The easiest way is to determine the phoria at a given fixation distance and then add a plus or minus lens before the fixing eye and again measure the phoria. The amount of change in the phoria (convergence) brought about per diopter change in accommodation gives the AC:A ratio. This ratio may be useful clinically in problem cases in which symptomatic heterophorias must be dealt with, especially in early presbyopia, in other near-vision problems, and also when some insight into the magnitude of the accommodative innervation is desired.[1]

General aspects of accommodative convergence

Experience has shown that the AC:A ratio is essentially the same for the same person at all viewing distances and for any change in stimulus to accommodation, providing the retinal image is not too blurred. Of great importance, however, is the fact that the relationship between accommodative convergence and accommodation is linear throughout the entire range of response to changes in the accommodative stimulus; in other words, a unit change in the stimulus to accommodation always results in a corresponding specific amount of change in the convergence (phoria) within the entire range of ciliary response.

Some question exists as to whether the AC:A ratio should not be determined according to the actual change in accommodation rather than according to the change in the stimulus to accommodation. In our experience, however, it appears that it is the change in the stimulus to accommodation that gives rise to the change in accommodative convergence. This is borne out by the fact that the same linear relationship between the accommodative convergence and the powers of ophthalmic spherical lenses placed before the eyes (a given fixation distance) is maintained even when the powers of the lenses are such that the images are so blurred that the test details barely can be discriminated. This point of view also is borne out by the fact that a linear AC:A ratio can be found in absolute presbyopes, although the range of lens powers within which a response can be found is small (± 0.75 diopter). These results would imply, therefore, that it is the innervation to the ciliary bodies—whether the crystalline lenses have or have not responded fully—that gives rise to the accommodative-convergence synkinesis. Fincham[2] has suggested that only when out-of-focus blurring is greater than about 1.25 diopters will the eye fail to respond to the blur for an accommodative change.

Considerable evidence exists that the AC:A ratio is fairly constant for the same person and perhaps changes little with age. In certain of our own studies, the AC:A measured on one person daily over a six-week period showed a mean value of 3.62^{Δ}/D., with a standard deviation of only 0.25^{Δ}/D. However, this is a relatively short period

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from which to generalize. One should not be dogmatic with regard to the constancy of the AC:A ratio, for on occasion changes have been measured. On the other hand, deliberate efforts to change the AC:A ratio have not proved particularly successful. The ratio can be greatly increased when the eyes are under the effect of cycloplegic drugs (homatropine), and the magnitude of the increase appears to be related to the degree of cycloplegia.[3,4] Most important in this regard, however, is the fact that a linear relationship persists between the change in accommodative convergence and the change in stimulus to accommodation, within the range of blurring where the subject can discriminate the test details, although the ratio itself is greatly increased. Contrariwise, the use of pilocarpine and physostigmine apparently produces no statistically significant change in the AC:A ratio and, furthermore, linearity is again found.[5] It is possible that di-isopropyl fluorophosphate (DFP) may decrease the ratio.[6]

In complete presbyopia, there is some evidence that the AC:A ratio is also normal, although some of our recent data suggest that a higher AC:A ratio may be the rule. Even here, however, a linear response is found over the small range of change in stimulus to accommodation possible before the image blur is too great. For this discussion, however, the important aspect is the linearity of the data.

Some evidence has been brought forward that many patients with clinically significant convergence insufficiency also have a lower AC:A ratio.[1] Also, the AC:A ratio tends to be higher in myopes and in those patients with a greater esophoria at near than at distant fixation distances. In all these instances of increased or decreased ratios, however, the relationship continues to be linear over the range of response.

The possibility of a nonlinear response of accommodative convergence to changes in the stimulus to accommodation has been suggested by Westheimer,[7] principally but not entirely on theoretic grounds. As he pointed out, it is difficult to understand how such a linearity is maintained in view of the complex nature of both the accommodative and the convergence processes. In certain of our own data[1] obtained by measurements with Maddox rods at a viewing distance of 40 cm., some persons increasingly failed to respond fully to the change in stimulus to relax accommodation, as when plus lenses of increasing power were placed before the eyes, so that in this region the AC:A ratio was reduced. However, these same persons all responded linearly when negative lenses were used to increase the stimulus to accommodation. In our studies using the fixation-disparity technique on about 250 patients, only about eight percent have shown definitely this lack of response to plus lenses when tested at a near viewing distance.

Fixation disparity as a measure of oculomotor imbalance

The technique of using fixation disparity to measure oculomotor imbalances may be a more reliable method than is that of the Maddox rod, since both eyes are subject to the same stimuli of the target, the same level of light adaptation, and the same stimulus to accommodation; in addition, fusion is maintained, and both eyes are subject to the same task in the measurements. This technique has been described fully elsewhere.[1,4,5,8] It will be only briefly reviewed here, principally to make clear the method of obtaining the data with which the study of accommodative-convergence synkinesis has been made.

The phenomenon of fixation disparity rests on the fact that in binocular vision with fusion, when an oculomotor imbalance exists, the eyes will actually deviate from exact convergence in the direction of the phoria. This small angle between the actual convergence and the convergence required by the fusion detail is the fixation-disparity angle and as such is a measure of the oculomotor imbalance. It (or a quantity proportional to it)

can be measured under suitable conditions.

Figure 1 shows schematically the test target with which fusion is maintained over a large field but prevented at the center. At this center, two vertical lines are arranged so that the lower one is seen by the right eye only, the upper one by the left eye only. The position of one of these lines can be varied. In the test, this is adjusted until the two lines appear to the subject as being aligned. The small actual separation of the lines is the fixation disparity, and this is expressed in minutes of arc of angle subtended by the eyes. For the present study, the test was conducted at 40 cm.

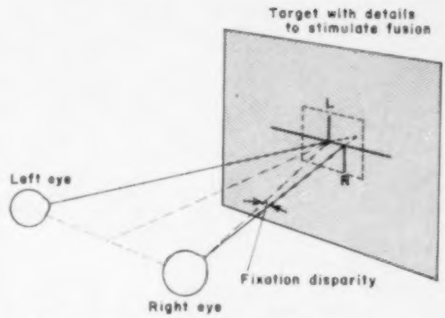

Fig. 1 (Martens and Ogle). Scheme for the measurement of fixation disparity.

The procedure is to measure the fixation disparity under two conditions. The first measurements are made when the eyes are forced to convergence and diverge by placing a series of prisms before the eyes, alternately base out or base in. In this process, a constant stimulus to accommodation is provided and the oculomotor imbalance is altered by changing the required convergence for fusion relative to the phoria position of the eyes. The second measurements are taken when the stimulus to accommodation is changed by placing a series of plus or minus ophthalmic spherical lenses before the eyes. In this process, a constant convergence for the 40-cm. distance is provided, but the oculomotor imbalance is altered by changing the phoria position of the eyes by virtue of the change in accommodative convergence. When these two sets of data are appropriately plotted, one obtains graphs typified by those shown in figure 2-A and-B.

The relationship between the accommodative convergence and the change in the stimulus to accommodation then is derived as shown in figure 2-C. In this graph, the abscissas are the same as those of Figure 2-B, namely the changes in the stimulus to accommodation—an increase (minus lenses) to the right, a decrease (plus lenses) to the left, in diopters. However, the ordinates now are the equivalent degrees of increased forced convergence, with prisms base out (upward), or of increased divergence, with prisms base in (downward), in prism diopters—a scale corresponding to that of the abscissas of Figure 2-A. The data points are obtained by finding for each of the series of lenses used, as in Figure 2-B, that combination of prisms, interpolated on the curve in figure 2-A, which gives the same fixation disparity. For example, in Figure 2-B, the fixation disparity measured when −2.00-diopter lenses were placed before the eyes was four minutes of arc eso-disparity.

In figure 2-A, the same fixation disparity was produced when prisms of a total deviating power of 7.5 diopters base-in were placed before the eyes. Thus, in Figure 2-C, for the increased stimulus to accommodation of 2.00 diopters, the equivalent (7.5 prism diopters) is plotted on the ordinate in the minus direction and indicated by an x. This procedure is carried out for all lenses used.

By this technique, the actual change in the phoria produced by a change in the stimulus to accommodation is not determined directly but is measured in terms of the change in the oculomotor imbalance produced by the lenses. Consider that a given person is orthophoric for the test distance, as is essentially true for the person whose data are illustrated in Figure 2. If lenses of −1.00 dioptric power are placed before the eyes, there will be an accommodative convergence of 3.3 prism diopters, and if one eye is covered by a Maddox rod one can measure

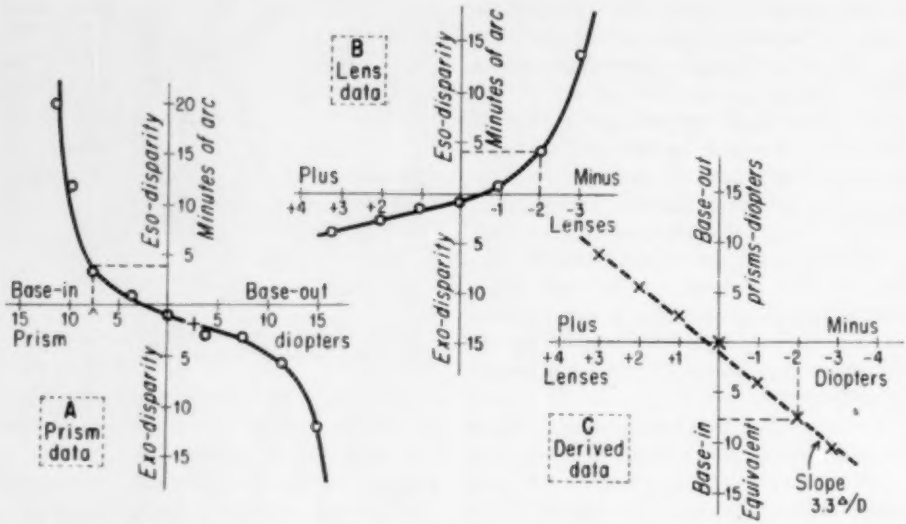

Fig. 2 (Martens and Ogle). Graphic representation of fixation-disparity data for a normal orthophoric person. (A) Curve for prisms. (B) Curve for lenses. (C) Derived data showing relationship between change in stimulus to accommodation (lenses) and the equivalent prismatic deviation.

the esophoria produced by placing a prism of 3.3 prism diopters base out before one eye. When fusion is maintained, however, this phoria is overcome, but the oculomotor imbalance remains. Now, relative to the phoria convergence position of the eyes due to the increased stimulus to accommodation caused by the −1.00-diopter lenses, the convergence (in this case a relative divergence) required by the target would correspond to prisms placed before the eyes of 3.3 prism diopters base in. Thus, relative to the phoria convergence position of the eyes, a relative divergence of 3.3 prism diopters is required if fusion is to be maintained. The derived data in the graphs C in the figures to follow thus correspond to this point of view.

These data when plotted in graph C usually fall on a straight line. The slope of the line that best fits these data points is equivalent to the AC:A ratio. The fact that the derived data points fall on a straight line throughout the range of response is the evidence for the linear relationship of the accommodative-convergence synkinesis to the stimulus of accommodation.

Present studies

The great majority of our series of about 250 patients on whom the accommodative-convergence synkinesis was studied by the fixation-disparity technique showed derived data that were linear throughout the range of response to power lenses, that is, up to the point at which either diplopia occurred or the images were so blurred that the patient could not discriminate the details of the test target. This result in so many patients is somewhat remarkable.

However, as already indicated, about eight percent of the patients studied showed a significant nonlinearity in the AC:A (stimulus) response to changes in stimulus to accommodation. It is the purpose of this paper to discuss these cases with regard to the nature of this nonlinearity and with regard to the results of the general ocular examinations. These people were for the most part regular patients at the Mayo Clinic in whom the ocular studies were only a part of their general medical examination.

At the outset, it was clear that a consistent pattern in the nonlinearity of the AC:A re-

sponse generally was absent. Figures 3 through 7 illustrate types of such nonlinearity found. In these graphs, the data are graphically illustrated in the following order from left to right: disparity-prism data (A); disparity-lens data (B); the equivalent prism-lens (derived) data (C). The reader may refer to Figure 2 for the respective designations used for the co-ordinate systems. In this paper, we are primarily interested in the graphs labeled C, the derived prism-lens data, for it is in these that the nonlinear relationships are to be seen. The equivalent AC:A ratio for the lenses of given power placed before the eyes will be the slope of the line tangent to the curve at that graphic point.

Figure 3 illustrates data on three patients in whom the equivalent AC:A ratio decreased as plus lenses of increasing power were used before the eyes. There were no unusual features in the disparity-prism or disparity-lens curves except that the response to plus lenses of increasing power in all three

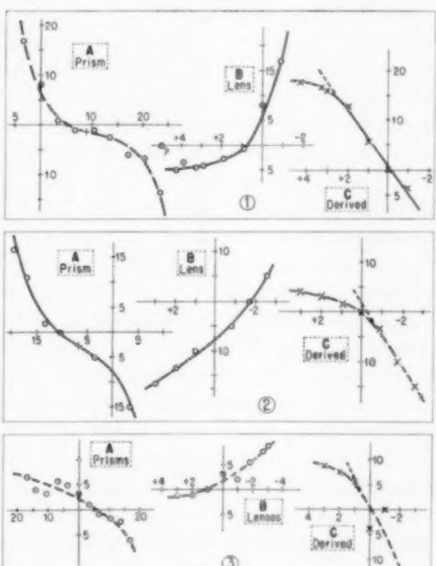

Fig. 3 (Martens and Ogle). Examples of a nonlinear AC:A relationship in which the relationship is decreased when plus lenses are used to induce a relaxation of accommodation.

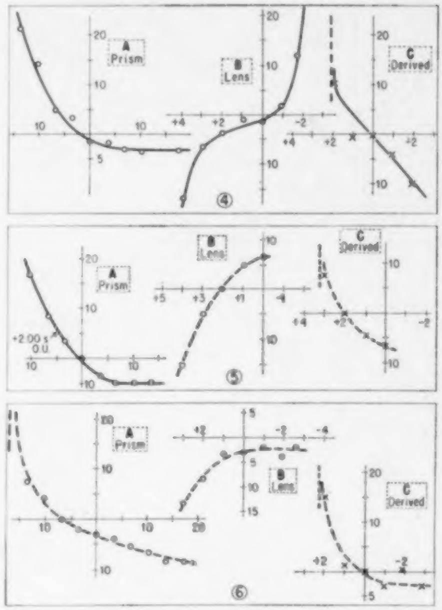

Fig. 4 (Martens and Ogle). Examples of a nonlinear AC:A relationship in which the relationship is increased when plus lenses are used.

produced a decreasing oculomotor imbalance, that is, a smaller accommodative-convergence effect was noted.

Only eight of the nearly 250 patients studied by this technique followed this pattern with certainty. However, in a previous study[1] on 30 patients, in which the Maddox rod was used to measure change in phorias, nearly half exhibited in part this failure to respond fully to plus lenses, although all responded linearly to minus lenses. The difference in the results of these two studies may point up the value of the fixation-disparity method.

Figure 4 illustrates data on patients showing the reverse pattern, that is, an increasingly greater equivalent AC:A ratio occurred for plus lenses of increasing power up to the point at which the ratio became indeterminate. Inspection of the basic data curves (A and B) shows that in these patients either the plus lenses of increasing power caused proportionally greater exophoric oculomotor

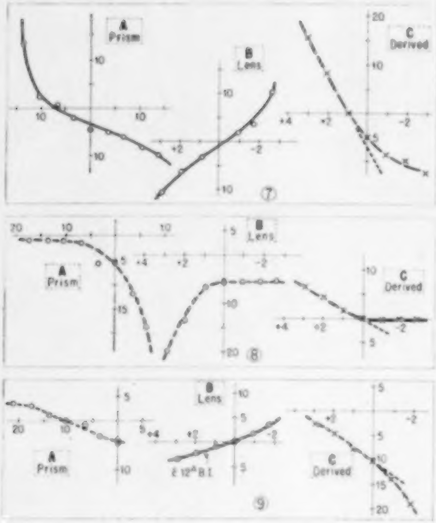

Fig. 5 (Martens and Ogle). Examples of a nonlinear AC:A relationship in which the relationship is decreased when negative lenses are used to induce an increase in accommodation.

imbalance or the use of prisms base-out of increasing deviating power resulted in a progressively decreased oculomotor imbalance. The equivalent forced convergence that produces the same oculomotor imbalance becomes increasingly greater. It is found frequently that lenses before the eyes actually may produce a greater oculomotor imbalance by virtue of accommodative convergence than does any convergence forced by prisms. This was true in these patients.

In Figure 5, the data illustrated in cases 7 and 8 show the reverse effect, in which the stimulus to increased accommodation resulted in a decrease in the equivalent AC:A ratio. In Case 8, in particular, the derived ratio was substantially zero for minus lenses. Inspection of the basic data (A and B) in this case shows that both the increased stimulus to accommodation and the forced relaxation of convergence resulted in a decreased oculomotor imbalance. At no time was it possible to cause an esophoric oculomotor imbalance by either lenses or prisms. This patient showed, by usual tests, little capacity to accommodate. In Case 9 in this same figure, the equivalent AC:A ratio increased with increased stimulus to accommodation (minus lenses). This particular patient showed a partial paralysis of convergence; thus, in order to obtain the disparity-lens data, it was necessary to place prisms base-out of 6^{Δ} prism power before each eye. With the increased stimulus to accommodation, the esophoric oculomotor imbalance became increasingly greater in contrast to that produced by forced relaxation of convergence (prisms base-in). The data in Case 10 (fig. 6) show an even more exaggerated situation in which the equivalent AC:A ratio became indeterminate for minus lenses greater than 1.00 diopter. It is clear that the increased stimulus to accommodate under a fixed convergence rapidly increased the oculomotor imbalance in this instance. This patient exhibited an esotropia at both distant and near vision after occlusion of one eye.

In Figure 6, the data in Cases 11 and 12 illustrate situations in which the equivalent AC:A ratio decreased for both increased and decreased stimuli to accommodation (minus and plus lenses). This result must be attributed to the pronounced dissimilarity between the basic data curves A and B. In Case 11, the patient exhibited esotropia at near vision after the cover test. In Case 12, the patient reported that he had had intermittent exotropia as a child but that this had decreased in later years.

The data illustrated in Figure 7 show examples of the reverse pattern, in which the equivalent AC:A ratio increased rapidly for both increase and decrease in the stimulus to accommodation, until points of indeterminancy were reached. Inspection of the basic data in Cases 13 and 15 shows disparity-prism curves, encountered less frequently, in which the oculomotor imbalance did not increase when the eyes were forced increasingly to diverge (prisms base-in) or to converge (prisms base-out). The pattern

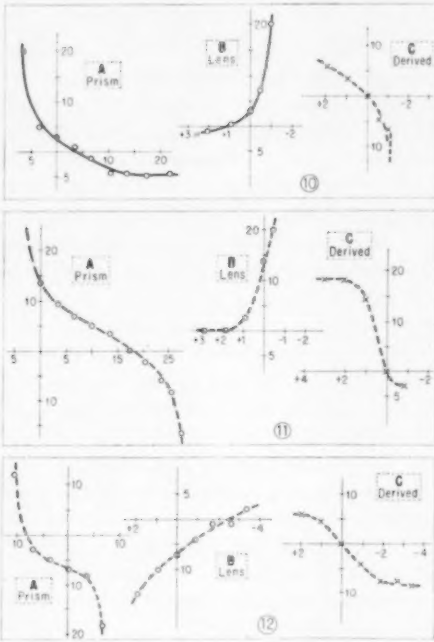

Fig. 6 (Martens and Ogle). Examples of a nonlinear AC:A relationship showing an increased relationship with negative lenses (case 10), and a decreased relationship for both plus and minus lenses (cases 11 and 12).

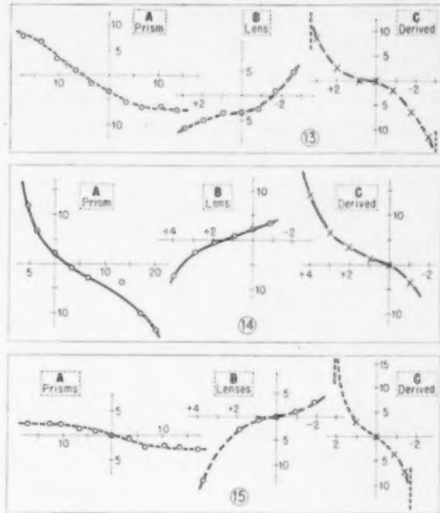

Fig. 7 (Martens and Ogle). Examples of a nonlinear AC:A relationship in which the relationship is increased for both plus and minus lenses.

of the resultant derived data curves undoubtedly results from this fact.

Comment

Attempts to relate the results of general clinical examination to the occurrence of these nonlinear AC:A sets of data have not been particularly rewarding. As a clinical impression, it does appear that, as a group, the patients falling into this class present more complicated problems of oculomotor co-ordination and asthenoptic symptoms than are presented by patients routinely. There also appeared to be a larger percentage of intermittent tropias, or tropias following the cover test. Patients exhibiting poor response to plus lenses invariably showed an abnormal phoria measurement, being esophoric (Maddox rod) usually for both near and distant fixation distances. Those patients who showed poor response to minus lenses always had a fairly large exophoria (Maddox rod) and symptoms of accommodative asthenopia associated with visual tasks in the reading range. Those patients who demonstrated an excessive response to minus lenses also showed an unstable fusional co-ordination and intermittent exotropia as demonstrated by certain clinical tests.

Unfortunately, it was not possible to retest many of the patients in this study, so we are uncertain as to the stability of this nonlinear accommodative-convergence response. For one patient (fig. 4, case 6), however, the AC:A nonlinear patterns of two tests, the second made four years after the first, were in excellent agreement. It has not been possible in these patients to obtain comparative data made with the Maddox rod for correlation with the data obtained by the disparity technique.

The chief point to be made in this paper is not to call attention to the direct relationship between particular clinical findings and the

occurrence of a nonlinear equivalent AC:A but to emphasize that (1) evidence exists that, in the great majority of patients, the AC:A ratio is linear throughout the range of response to change in stimulus to accommodation and (2) the equivalent AC:A ratio departs from this linearity in about eight to 10 percent of patients tested by the fixation-disparity technique.

We cannot easily raise the question as to why such nonlinearity occurs in this group of patients, for we do not yet understand why the AC:A relationship generally maintains a linear form in the majority of patients tested. In the nonlinear group, however, a fairly definite defect or abnormality apparently exists in one component of the fusional processes, which in all cases might be recognized by the clinical tests commonly employed. In the great majority of subjects it would only appear that, when the innervations proceed to the ciliary body in an effort to exert a given change in accommodation, a certain proportional innervation also goes to the "centers" for convergence.

Summary

The fixation-disparity method of determining oculomotor imbalance was used in about 250 patients to study the relationship between accommodative convergence and the stimulus to accommodation. The great majority of these patients showed a linear relationship between accommodative convergence and the changed stimulus to accommodation throughout the entire range of response to ophthalmic spherical lenses and, therefore, they showed a constant accommodative convergence:accommodation ratio. In about eight percent of these patients, however, the relationship was nonlinear in some respect. A general pattern of nonlinearity was not present. An attempt to relate the results of clinical examination by tests commonly employed to the nonlinear responses was not particularly rewarding, but sufficient evidence existed that, as a group, these patients presented more complicated oculomotor problems than are encountered routinely.

Mayo Clinic.

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Discussion

Dr. Gerhard A. Brecher (Atlanta): The results reported in this paper need to be looked upon from a broad view in order to do justice to their significance. The problem of the relation of accommodation to convergence is part of the fundamental physiologic problem of neuromuscular coordination. In this specific case there is one sensory input, the visual stimulus, which results in two motor outputs. From a neurophysiologic standpoint, this phenomenon can be called divergence of impulses. Both motor outputs are directed into entirely different effector areas which do not have many functional relations in any other respect. One area is represented by the smooth ciliary muscle and the other is represented by a group of striated muscles of the eyeball. The amazing feature is that in most persons such close correlation has been established between these motor organs which act otherwise quite independently. It is manifested by the finding that the relative increments of ciliary muscle contraction and extraocular muscle contraction are practically identical over a wide range of action. This is what one generally calls the linear relationship of the accommodation-conver-

gence ratio. It is much less amazing that this linearity is lacking in about eight percent of the examined subjects.

As the authors point out it is not yet understood why the accommodation-convergence relationship is generally linear. One can only assume that this particular neuromuscular co-ordination has developed during maturation of the organism in the same way as co-ordination appears to develop in other motor areas, that is, by facilitation controlled through feedback systems. Concluding from this general assumption and the data, one may state that in most persons this development has been accomplished successfully. The finely graded motor responses to impulse trains leading to the two different muscle areas have reached after the developmental stage a rather fixed relation. The linearity of this relation is the best approximation to the geometric optical necessities for convergence at near vision. The linearity of the response relationships will minimize the necessity of additional binocular fusion movements for accomplishing single vision. The eight percent of the subjects who show no linear relationship indicate that the development of their neuromuscular coordination has reached less perfection. This manifested itself in the authors' experiments insofar as these patients showed also unstable fusional co-ordination under some other clinical tests. The nonlinear responding subjects may then be considered as persons whose development of neuromuscular co-ordination might be further developed by suitable reinforcement. This opens up possibilities of improvement of neuromuscular co-ordination by systematic training. Of course, such training would be most effective in childhood.

The question arises why the authors did not establish more precisely the interesting relation of the nonlinear accommodation-convergence response to muscle imbalances detectable with other methods. This would have involved a very elaborate investigation with repeated and detailed studies of each of the "nonlinear" subjects. It was obviously beyond the scope of this study which consisted of data accumulation by screening many subjects. The value of the present contribution is to have pointed out the direction in which future work still needs to be done. The method of fusion disparity proved to be a reliable and useful tool for accommodation-convergence determinations, which was applicable to a large group of untrained observers. This method could be easily used for an extension of these studies in which the test conditions may be varied, for example, it would be of interest to find out whether or not the "linear" subjects exhibit the same linear relationship at secondary or tertiary eye positions or at asymmetric convergence. Vice versa, it could well be that some "nonlinear" subjects show a linear relationship if examined at different eye positions. Detailed studies of this type with the fusion disparity method may reveal with great precision muscle imbalances which cannot be so easily detected with other more conventional methods.

Dr. Henry Knoll (Los Angeles): The authors, in addition to presenting some very excellent data, have in this paper and in previous papers raised the question as to how we are going to define the AC:A ratio. If I remember correctly, the AC:A ratio is defined initially in terms of the stimulus in both cases, the stimulus to accommodation and the stimulus to convergence.

Subsequently, Fry and Morgan and others have reported the AC:A ratio in terms of the response of accommodation and the stimulus to convergence. These data now present the stimulus to accommodation and the response of convergence as measured by the fixation disparity method.

There are some data forthcoming which will present the AC:A ratio, in which the accommodative response and the convergence response have been measured, or at least such an attempt has been made. So, there are any number of combinations and permeations that it is possible to introduce here.

In the disparity method that Ogle has used, the actual stimulus distance is maintained constant, changing the accommodative stimulus by changing the plus or minus lenses. This is another parameter, then, as to whether we are going to actually move the target in space.

I don't think any of us is in a position to say which is the AC:A ratio. All we can say is that here are some AC:A ratio data taken by such-and-such a method.

Certainly we would like to find a method which most nearly represents the true neurologic picture, but whether we can find that is another question.

I would like to add to Dr. Brecher's comments that there are actually three responses to this single stimulus. The third one, of course, is the pupil, and it has been demonstrated that this is also very nicely and in many cases linearly related to the accommodative stimulus.

Dr. T. G. Martens (closing): This is not entirely in the realm of physiologic research. I am sure that some day this fixation-disparity method of testing ocular motor imbalances will come down to a clinical level where it is easily applicable.

Rather interesting phenomena are encountered every once in a while. As an example, the patient who has puzzling asthenopic symptoms and who is found to have an exophoria may actually, with fixation-disparity techniques, be found to have an esophoria, purely descriptive of what actually is going on when fusion is in existence and you are not shutting out the normal binocular reactions during the test.

THE EFFECTS OF AUTONOMIC DRUGS ON HUMAN FLICKER DISCRIMINATION*

Mathew Alpern, Ph.D., and Robert S. Jampel, M.D.
Ann Arbor, Michigan

Introduction

Wachholder and Arnold,[1] Wachholder and Schneider,[2] and Fleming[3] have obtained variation in the critical flicker frequency threshold[4] by injection (or ingestion) of autonomic drugs. Fleming[5] has also found that many of these same drugs when instilled into the conjunctival sac would reproduce these effects in the eye to which the drug was applied but not in the other eye. He showed that sympatheticomimetic and parasympatheticolytic agents produced an increase in the critical flicker frequency while sympatheticolytic and parasympatheticomimetic agents produced a decrease. The general conclusion of these studies is that such effects are largely due to modifications of retinal efficiency as a result of disturbances in the balance of the two components of the autonomic nervous system as they "steer" the retinal function.[1]

It is important to be certain that the effects described cannot be accounted for on the basis of more obvious factors. In this respect the influences of comparative miosis and mydriasis following instillation of the drugs are of prime interest. These influences are both obvious and subtle. A wide pupil allows more light to reach the retina and as a consequence of the well know Ferry-Porter relation this in itself will elevate the critical flicker frequency.[6] Conversely, a small pupil will reduce it. Less apparent is the influence upon the state of adaptation of the eye in Fleming's procedure. The method is to measure the threshold alternately in one eye and then the other. The effect of the drug is determined by the difference in the critical flicker frequency in the two eyes. If the drug produces mydriasis then the drug eye will be more light adapted, if the drug is a miotic that eye will be relatively dark adapted, as compared to the control eye. The effects of light and dark adaptation on critical flicker frequency have been especially emphasized by Lythgoe and Tansley.[7]

In addition, the possibility remains that the drugs might influence the extraocular muscles rather than the retina. The eyes are never at rest[8] even in the steadiest of fixations and autonomic drugs, which admittedly influence extraocular muscle activity,[9] could conceivably influence the frequency and amplitude of these fine oscillatory movements. If the target is a small 41-minute object seen against a completely dark surround (as was indeed the case in Fleming's experiments) then these oscillatory movements could conceivably influence the flicker thresholds by changing the adaptation state of the region of the retina to which the test stimulus was applied.

The previous investigators were obviously aware of some, if not all, of these factors. However, study of their work emphasizes the fact that the theoretical conclusions are based upon evidence in which these possible contaminating influences have not been controlled carefully enough. It therefore seemed important to study the effects of autonomic drugs upon flicker discrimination under conditions in which as careful as possible control could be made of the influences of pupil size, adaptation, and eye movements upon the results. For this purpose the most critical experiments, of instillation of the drug into the conjunctival sac of one eye and making alternate measurements of the drug and control eye, were employed. Pupil size influences were obviated by using a very small (1.4 mm. in diameter) artificial pupil. This was always considerably smaller than the smallest measured entrance pupil size. Eye movements

* From the *Department of Ophthalmology and Vision Research Laboratories, The University of Michigan.* It is a pleasure to acknowledge the support of this work by the Knight's Templar Eye Foundation, Inc.

effects were avoided by using a large circular (two degrees in diameter) test object which was surrounded by an annulus of outside diameter of six degrees. The luminance of the annulus (except at levels higher than about 20 c./m.²) was always equated to the Talbot luminance of the flickering field and the observer was instructed to fixate always at the center of the test field. Adaptation was controlled by maintaining such fixation for about one minute prior to the beginning of a measurement with the eye about to be tested. When low levels of illuminance were studied, careful dark adaptation was carried out for 30 minutes prior to the adaptation to the luminance of the field. The eye not being tested was screened from any stray light.

Apparatus and methods

The apparatuses (fig. 1) used in these exepriments are all fairly standard for making measurements of these sorts and are well described in the literature.[10-13] A brief description only will be outlined. To measure critical flicker frequency a 32 candle-power tungsten filament headlight lamp was connected to a well charged six-volt battery. An image of the lamp filament was focused upon a small two mm. in diameter circular diaphragm and this diaphragm was imaged in turn upon the sectors (on/off ratio of unity) of a sturdy cardboard disc. The disc was mounted to a shaft connected to a variable speed friction drive which permitted the rate of rotation of the disc (and therefore the rate of alternation of the flickering light) to be varied. The light emerging from the open sectors of the disc illuminated an opal glass screen which was viewed with aid of a convex lens through the field of a Macbeth Illuminometer. The central part of the photometer field served as the flickering target; the surround was the measuring light of the illuminometer. The artificial pupil was centered over the end of the illuminometer and the observer placed his eye as close to the pupil as he could. Suitable neutral density filters could be placed both in the flickering field and over

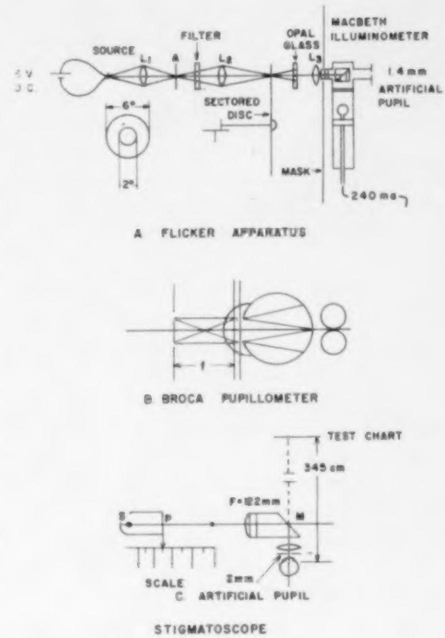

Fig. 1 (Alpern and Jampel). Apparatus used in these experiments.

the steady surround to vary the intensity of either independently. The lamp was allowed to heat up for 30 minutes prior to the beginning of any experiment. In this way the luminous output of the lamp was stabilized. Nevertheless, the apparatus provided an ideal system for a constant monitoring of the intensity of the flickering field. Even the slightest variation in this latter was immediately detected and proper adjustments for it could be instituted. Moreover, the procedure of alternate measure of drug eye against its normal fellow not only insured against the influences of temporal variation in luminance of the stimulus but against those of temporal variations in the physiologic and psychologic state of the observers as well.[13]

The rate of rotation of the sectored disc was measured with a tachometer (revolution counter) and stop watch in early experiments. Later a General Radio Company

Strobotac was used for this purpose.

Pupil size was measured using the Broca pupillometer. Two small pinholes were placed in the anterior focal plane of the eye. The separation of the pinholes was varied until the observer saw two barely contiguous entopic images of his own exit pupil. The separation of the pinholes necessary to produce this effect was a measure of the size of the entrance pupil.

Ametropia and/or accommodation disturbs the validity of such measurements so that they must be regarded as only approximate. In the case of accommodation (such as resulted from the crucial case of parasympatheticomimetic agents) the increase of refraction of the eye brought the parallel light to focus in front of the retina so that when the correct separation of holes was placed in the anterior focal plane the entopic exit pupils appeared separated. Thus the measurements were in error in such a way that the measured diameters were smaller than the actual entrance pupil diameters. In such cases, however, the important point to be certain of was that the entrance pupil was larger than the artificial pupil. Consequently this error in measurement of pupil size in this limiting case was in the direction of providing a safety factor assuring that if the measured entrance pupil size was larger than the size of the artificial pupil, the actual entrance pupil size was even larger!

In hyperopia corrected by spectacle lenses (which was the case for one of our observers —M. A.) the error of this method of estimation of pupil size unfortunately was in the opposite direction. For the magnitude of ametropia under consideration (approximately 3.00 diopters in the meridian being measured) these effects were small, however, and were fortunately more than compensated for by the much larger effects of the increased cyclotonia at the crucial moments in the only relevant experiments. The errors in the estimation of pupil diameter by these means were very small and for all practical purposes they may safely be neglected. Consequently, no essential differences in these measurements were found between the hyperopic observer on the one hand and the emmetropic one on the other.

To estimate the status of ciliary activity measurements were made by setting a point source of light mounted as a stigmatoscope in a Badal optometer arrangement viewed by reflection in a half silvered mirror placed before the eye. The observer looked through a two mm. in diameter artificial pupil and any correcting spectacle lenses and by transmission in the mirror observed an ordinary ophthalmologic letter chart at the other end of the room. The stigma was seen as flashing (one second in every two) upon the chart and the observer moved the light source back and forth along the optical bench until it was seen in exact focus. From a knowledge of the optical characteristics of the optometer lens it is possible to compute the position of the conjugate focus of the retina if the position of the light source has been determined. The observer made such settings by "relaxing" as much as possible and staring off at the letter chart.

To determine the critical flicker frequently a modified method of adjustment was employed. The experimenter varied the rate of rotation of the sectored disc until the observer reported it as either "flicker" or "fuse." The experimenter then reversed the direction of rotation until the observer reported either "fuse" or "flicker" respectively. The experimenter then reversed the direction of rotation a second time and repeated the process gradually narrowing the gap between the two limits. After four or five such reversals the final setting was made, the end point always being the point of transition from "fusion" to "flicker." Recent experiments emphasize that such a procedure is much more precise and free from experimental artefacts than other techniques.[14]

Results

Part I. The influence of various drugs on flicker thresholds

In these experiments a statistical test was applied to determine if various drugs produced an effect on flicker. The procedure was to duplicate the concentrations, frequency of application, and drugs used by Fleming[5] and to make measurements at a time when he obtained maximum effects. For the drugs used in the present experiments (regitine 5.0 mg./cm.[3], epinephrine 1/1,000, homatropine hydrobromide, one percent, and eserine, one percent), he found maximum effects 30 minutes after the application of the drugs. Accordingly measurements were begun at this time and continued for 16 measurements immediately thereafter on each eye. The entire procedure lasted a little over one hour. Each drug was tested in this way twice on separate days on each of two observers so that a total of 32 comparisons were made on each observer. The Talbot luminance of the flickering stimulus provided a retinal illuminance of approximately 10 trolands.* The results of these experiments are given in Table 1. It is clear from this table that homatropine, regitine, and epinephrine had no significant effect on flicker thresholds when measured in this way. One-percent eserine, on the other hand, produced a decrement of about 1.4 cycles/sec. in the flicker frequency in each observer and these results are statistically quite significant.

The unlikely possibility existed that the results obtained were unique to an intensity of 10 trolands. To determine whether or not this was the case measurements were instituted at a variety of different luminance levels. Measurements were made after careful adaptation to the luminance level of the field and again begun only 30 minutes after the drug was instilled. For this (and only this) experiment control measurements were made on the same eye and on different days from the measurements with drugs. The results for observer R. S. J. are plotted in Figure 2 which shows the critical flicker frequency (cycles/sec.) on the ordinate as a function of the logarithm of the retinal illuminance (in trolands) on the abscissa. Each plotted point is the mean of three repetitions of the experiment, carried out on different days. The normal (control) data show that, for the range of intensities studied, the Ferry-Porter relation

$$\text{c.f.f.} - k \log I + b, \qquad \text{(eq. 1)}$$

in which I is the intensity of retinal illuminance and k and b are constants, accurately describes the result for this observer. The line which is defined by this equation is drawn in the extreme left of Figure 2. In order to present the maximum amount of information on the chart the data for the

* The amount of retinal illuminance in trolands is determined by multiplying the luminance of the target (candles/m.^{2}) by the area of the pupil (mm.^{2}).

TABLE 1

Effect of drugs on critical flicker frequency

Drugs	M.A.+	R.S.J.●	Drug E-Control		t	
	L.E.-R.E.	R.E.-L.E.	M.A.	R.S.J.	M.A.	R.S.J.
Control	−0.20	−0.27				
Regitine (5 mg./cc.)	+0.14	+0.17	+0.34	+0.44	1.39	0.986
Epinephrine (1/1000)	−0.26	+0.20	−0.06	+0.48	0.17	1.38
Homatropine HBr (1%)	−0.53	+0.31	−0.33	+0.58	1.21	1.84
Eserine (1%)	−1.36	−1.38	−1.16	−1.11	2.40▲	3.28■

\+ Drug in left eye.
● Drug in right eye.
▲ $P<0.05$.
■ $P<0.01$.
N = 32.

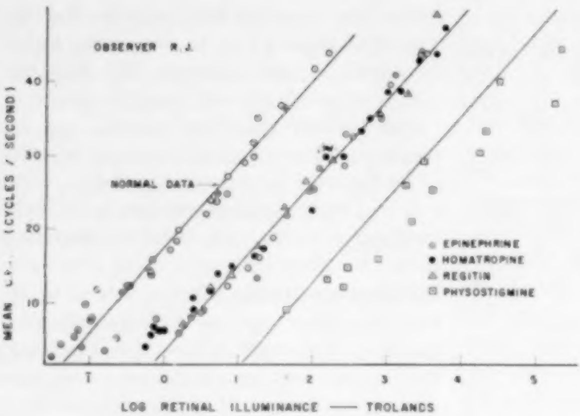

Fig. 2 (Alpern and Jampel). Effect of various drugs on critical flicker frequency at various levels of retinal illuminance (Talbot). Measurements were begun approximately 30 minutes following drug instillation and continued for about one hour thereafter. The straight line is the empirical fit of the Ferry-Porter law to the normal data. In the case of epinephrine, regitine, and homatropine, the data and the line have been shifted arbitrarily to the right one and one quarter log units. In the case of eserine the data and the line have been shifted arbitrarily two and one half log units to the right.

regitine, homatropine, and epinephrine experiments have been arbitrarily shifted approximately one and one quarter logarithmic units to the right, while the data for the eserine experiments have been arbitrarily shifted about two and one half logarithmic units to the right. All three straight lines represent the curves obtained by fitting (eq. 1) to the normal data, the two to the right being arbitrarily shifted the appropriate amounts.

The data in Figure 2 show clearly that homatropine, epinephrine, and regitine have no effect upon the critical flicker frequency threshold at any intensity level (over three logarithmic units) studied. One-percent eserine, on the other hand, seemed to reduce the magnitude of the critical flicker frequency about one or two cycles/sec. at each intensity level studied. There is, however, a considerable variability in these latter data, more in fact than is justified by the precision of the measurements involved. This suggested the possibility that the curves obtained by Fleming for the variation in flicker threshold following the application of the drugs were so distorted by artefacts of pupil size change, adaptation, and/or eye movements that time characteristics of the isolated effects on critical flicker frequency were considerably different from those he reported.

To examine this possibility, the measurements were made at about 10 trolands of retinal illuminance as a function of time following the instillation of the drugs. The results of these experiments, in the case of epinephrine, regitine, and homotropine for observer M. A. are illustrated in Figure 3. In the figure the abscissa values are plotted as time following the instillation of the drug while the ordinates show the difference between the critical flicker frequency measured in the drug eye and that of the normal control. These data emphasize, for the third (and last) time, that regitine, epinephrine, and homatropine have no effect on the critical flicker frequency under these rigorously controlled conditions. Clearly the results obtained by Fleming must be attributed to artefacts related to the variation in pupil size, adaptation, and/or eye movements as outlined in the introduction.

In direct contrast to these results were the effects of eserine, illustrated in Figure 4. Instillation of the drug to the left eye was follower after some minutes by a sharp fall in the critical flicker frequency in this eye. The effect reached a maximum after 15 minutes. The flicker sensitivity in this eye then began to rise and approached the normal value some 45 minutes after the drug was instilled. It is interesting to point out that the shape of this curve is quite different

from that obtained by Fleming for the same drug and these differences are probably attributable to the experimental artefacts in his methodology.

Figure 5 demonstrates that essentially the same kind of curve may be obtained at a wide variety of different intensities of the flickering stimulus following topical application of one-percent eserine. The data in these curves were the means of three repetitions of the experiment at each intensity level. The first point on each curve at each intensity shows the way the flicker threshold varies as a function of intensity for this observer. It is clear that the Ferry-Porter relationship was maintained for this observer, although as is usually the case the linear relationship begins to break down at higher light levels. The successive measures at each intensity level show the effect of one drop of one-percent eserine (placed into the conjunctiva of the left eye) for each five minutes following the instillation of the drug. These data were obtained by determining the difference between the drug and control eye in the usual

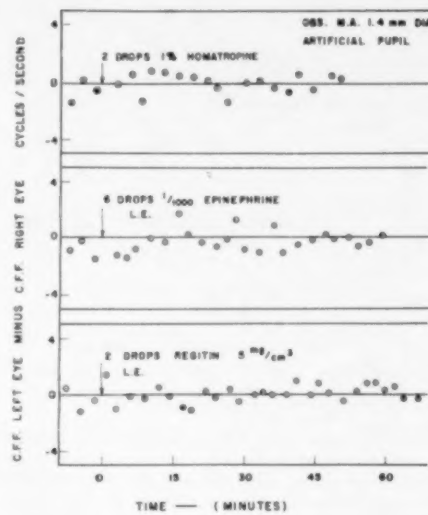

Fig. 3 (Alpern and Jampel). Effects of various drugs on critical flicker frequency at various time intervals following the instillation of the drug. The ordinate in this and many subsequent figures represents the critical flicker frequency of the normal eye subtracted from the critical flicker frequency in the drug eye.

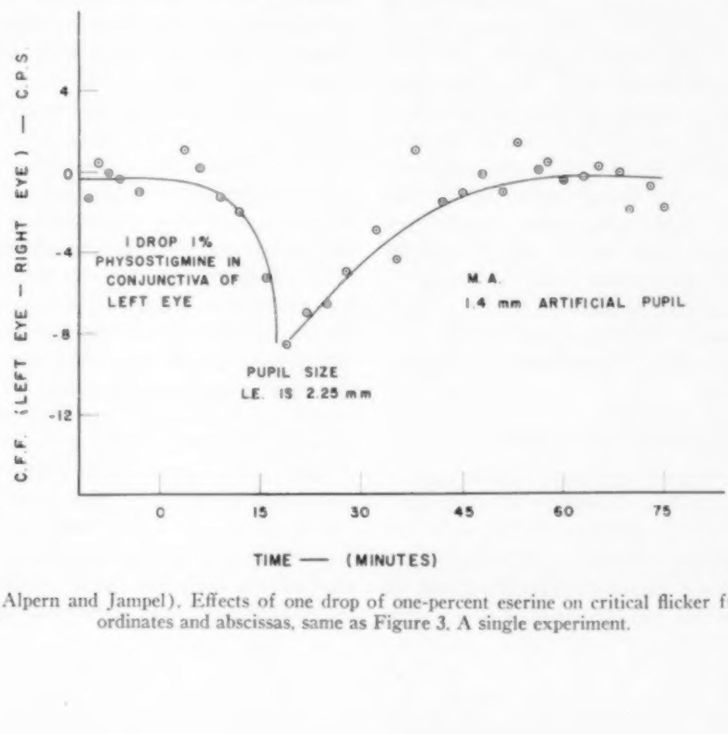

Fig. 4 (Alpern and Jampel). Effects of one drop of one-percent eserine on critical flicker frequency ordinates and abscissas, same as Figure 3. A single experiment.

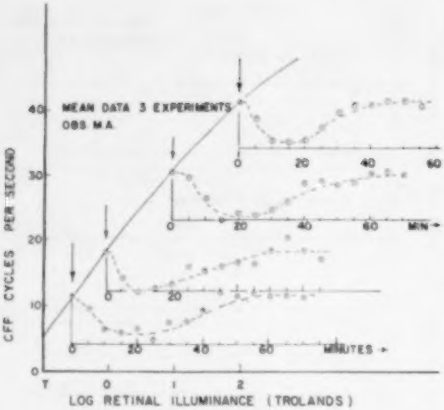

Fig. 5 (Alpern and Jampel). Effects of eserine on critical flicker frequency for various levels of retinal illuminance (Talbot) and for various time intervals following the instillation of the drug. Mean of three repetitions.

way and then relating the difference to the critical flicker threshold without any drug. For each intensity level the curve showing the decrements in flicker perception following eserine is essentially the same form. Individual differences between curves such as do appear are more probably related to random errors than to anything else. The important point seems to be that essentially the same absolute magnitude of decrement in critical flicker frequency occurs irrespective of the baseline level. Quite obviously this relationship must break down at the extremely low light levels where the absolute baseline level would be less than the magnitude of the decrements found in Figure 5. Perhaps a similar breakdown of the relation would occur at a high light level as well. Further experiments are required to elucidate these matters.

PART II. THE EFFECT OF ESERINE

The previous experiments have demonstrated that topical instillation of one-percent eserine had a pronounced effect upon the flicker threshold of the eye to which it was applied but not upon that of the other eye. To what may this be attributed?

In order to answer questions of this sort concurrent measurements were made of critical flicker frequency, pupil diameter, and the amount of approach of the far point of the eye following the instillation of the drug. Figure 6 illustrates the results of such experiments for two observers at each of three concentrations of the drug (1.0 percent, 0.5 percent, and 0.25 percent). These illustrated results are the means of three repetitions of the experiments (except for the results with 0.25 percent which are the results of single experiments). The results for both observers show the same general trends. The curves for pupil diameter run a consistently different course, following the instillation of the drug, than the change in flicker threshold and the approach of the far point (which closely parallel one another). When these facts are coupled with the fact that the entrance pupil diameter for each observer never was as small as the artificial pupil, then the miosis can be effectively ruled out as a factor in the production of the effects obtained. Weaker concentrations of eserine produce, quite naturally, weaker effects upon all three measurements but the effects upon flicker and the approach of the far point again follow one another quite closely.

The above observations suggest that it might be possible to block the action of eserine with some parasympatheticolytic agent. Experiments show that five-percent homatropine HBr applied topically to the conjunctiva has no measurable effect on the critical flicker frequency measured with the present flicker apparatus. Furthermore, when the homatropine was instilled concurrent with the eserine the same effect was obtained as if no homatropine had been instilled at all. On the other hand, when the homatropine was first instilled at least 30 minutes prior to the instillation of eserine then the effects of eserine upon flicker discrimination were effectively blocked. This result is illustrated for one of the observers in Figure 7 which shows the consequence of the instillation of five-percent or 2.5-percent homatropine HBr

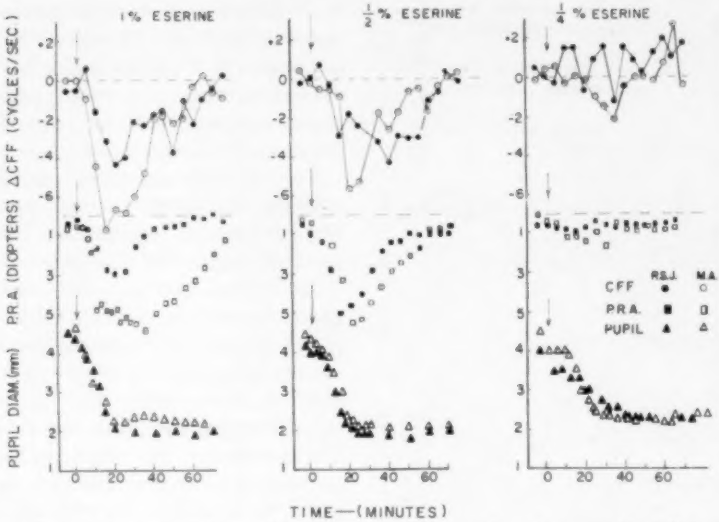

Fig. 6 (Alpern and Jampel). Effects of various concentrations of eserine on critical flicker frequency, pupil diameter, and far point of accommodation (diopters). The data for 0.25-percent eserine are from a single experiment. The remainder are the means of three repetitions.

30 minutes prior to the application of one-percent eserine upon subsequent measurements of critical flicker frequency, pupil diameter, and the approach of the far-point. In this graph each point is the mean of three repetitions of the experiments. As in the previous experiment the Talbot illuminance produced by the flickering stimulus was about 10 trolands. Five-percent homatropine virtually eliminates any effect of eserine upon flicker or upon the approach of the far point but even 2.5-percent homatropine has a measurable effect. Again the curves for the critical flicker frequency and the approach of the far-point are very similar while those for pupil diameter are quite distinctly different.

The results illustrated in Figures 6 and 7 suggest that the effect of one-percent eserine upon critical flicker frequency could be produced by the contraction of the ciliary muscle induced by the inhibition of cholinesterase. This might be brought about in one of two conceivable ways:

A. The contraction of the ciliary muscle by producing a relatively strong myopia could cause a fall in the critical flicker fre-

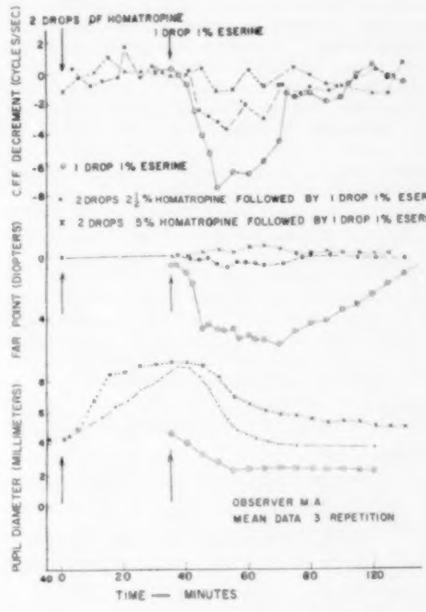

Fig. 7 (Alpern and Jampel). Instillation of homatropine 30 minutes prior to the instillation of one-percent eserine blocks the effect of the latter upon critical flicker frequency.

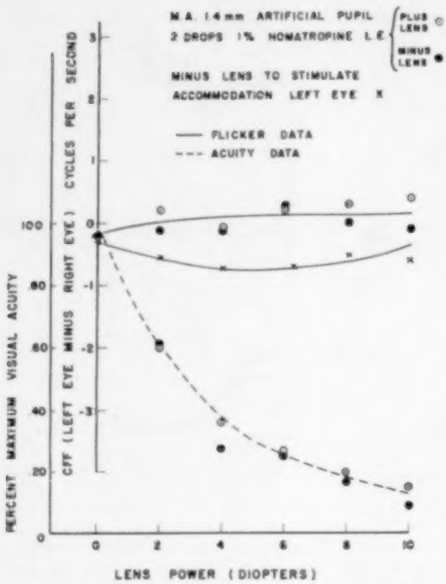

Fig. 8 (Alpern and Jampel). Effect of ciliary muscle activity upon critical flicker frequency. The solid lines represent flicker data; the dotted lines represent visual acuity data. The circles show the effect of minus lenses (filled) and plus lenses (open) when the ciliary muscle is paralyzed with homatropine. The separation of the solid and dotted lines shows the dissociation of flicker and visual acuity under these conditions. The X's represent the flicker thresholds determined by forcing the normal eye (unparalyzed ciliary muscle) to accommodate as much as necessary to the minus lens in order to see the test pattern sharply defined. In each case the critical flicker frequency was measured for the left eye looking through the trial case lens and this was compared to the critical flicker frequency of the right eye which was alternately measured as a control. Each point is the mean of five measurements. Visual acuity measurements were also carried out on the left eye of the same observer.

quency because of the resulting blur of the retinal image.

B. The contraction of the ciliary muscle by exerting stress upon the choroid could produce a mechanical traction upon the visual cells and in this way reduce their flicker sensitivity.

In support of the first hypothesis is the fact that measurements of visual acuity and the critical flicker frequency for the center of the field show a very high positive correlation (higher than 0.9) with each other.[15] It is known that a blurred image has a marked deleterious effect upon acuity[16] and one might expect that perhaps the same would hold true for critical flicker frequency.

To check this possibility, an effective cycloplegia of the left eye was first obtained with one-percent homatropine. Then various plus and minus trial case lenses were introduced before this eye and measurements were made of the critical flicker frequency of this eye as compared to that measured with the normal control right eye (which had no trial case lenses before it at all). Five measurements were made with each different lens. The results are illustrated in Figure 8 in which it is clear that, contrary to its effect upon visual acuity, blurring of the retinal image has no appreciable effect upon the critical flicker frequency. The dissociation of the correlation of visual acuity with flicker thresholds was further demonstrated by measuring the visual acuity with a variable-testing-distance-constant-size-letter chart procedure upon the same cycloplegic left eye with the various plus and minus trial case lenses. The latter curve is illustrated in Figure 8 as a dotted curve, the flicker results as a solid curve. Clearly the results obtained by instillation of one-percent eserine are not to be explained by the blur associated with the approach of the far point.

The hypothesis B suggested above was tested by carrying out the opposite experiment. In this no cycloplegia was produced but on the contrary the left eye was forced to accommodate as much as was necessary in order to see the fine structure of the target clearly as various powers of concave lenses were placed before the eyes. Five measurments with each lens were again compared with an equal number of measurements made alternately with the control right eye (no trial case lenses at all before it). The results are illustrated in Figure 8 by the X's. Clearly as the eye was forced to accommodate the

critical flicker frequency was reduced and this reduction increased as the stimulus to accommodation increased up to a maximum of about five diopters. Further increase in the stimulus to accommodation beyond this point produced less and less of an effect, perhaps because the accommodation system was no longer capable of keeping up with the stimulus. But even at its maximum the effect was a whole order of magnitude smaller than one obtains with a single drop of one-percent eserine and so it seems highly unlikely that hypothesis B above can explain the effects of eserine on critical flicker frequency either. This seems to imply that neither miosis nor ciliary muscle contraction can be responsible for the reduction in the flicker threshold produced by eserine.

Part III. The effect of pilocarpine

Eserine inhibits the action of the cholinesterase enzyme and thereby allows whatever acetylcholine is produced to have a prolonged effect. Acetylcholine, itself, has two effects: (a) an effect upon smooth muscle and upon glands which can be duplicated by muscarine and (b) an effect upon the motor end plates of striped muscles as well as an effect upon nerve synapses which can be duplicated by nicotine. To which of these effects of acetylcholine is the effect of eserine upon the critical flicker frequency more closely related? In order to answer this question measurements have been made of the effect of topical application of various concentration of pilocarpine upon critical flicker frequency, pupil diameter, and the approach of the far point. Pilocarpine, as distinct from eserine, mimics the action of acetylcholine on smooth muscles and glands, that is, it is a muscarine-like substance.

The results of a few such experiments are illustrated in Figure 9, which shows the effect of topical application of four-percent pilocarpine. Clearly, the known muscarine effects of acetylcholine on the eye were duplicated by pilocarpine but this latter drug

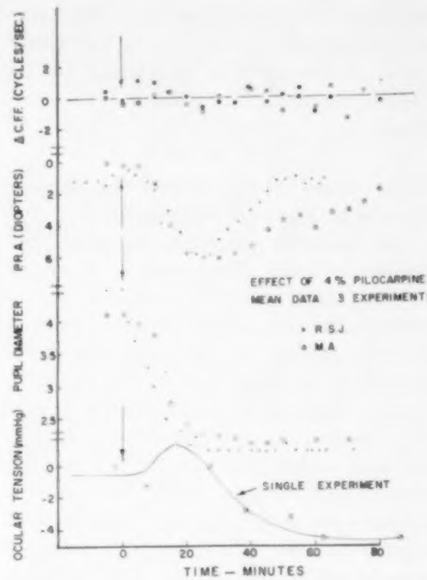

Fig. 9 (Alpern and Jampel). Effect of four-percent pilocarpine upon critical flicker frequency, pupil diameter, far point of accommodation, and intraocular pressure. The latter data were obtained with a clinical tonometer alternately placed on the drug eye and the normal control. The ordinate represents the tension in the normal eye (in mm./Hg) subtracted from the tension of the drug eye.

had no effect at all upon the critical flicker frequency. The same results were invariably found even with concentrations of pilocarpine as high as eight percent. In these concentrations pilocarpine had a greater effect upon accommodation and pupil size than does one-percent eserine.

In one experiment concurrent measurements were also made upon the intraocular pressure with a clinical tonometer. These results (the lower curve in fig. 9) showed a mild temporary increase in the tension of the drug eye as compared to the control. This is followed by a rather prolonged fall in the pressure of the drug eye. Essentially the same effects upon the intraocular pressure of the same eye have been found with one-percent eserine. Although only a very few such measurements have been carried out

they offer no reason for the belief that variations in ocular pressure following the instillation of eserine could be responsible for the observed decrement in critical flicker frequency.

Discussion

The results of experiments like those illustrated in Figure 9 emphasized that pilocarpine produced essentially the same effect upon the iris and ciliary muscles as eserine did. The latter, however, caused a marked reduction in the critical flicker frequency while the former did not affect the flicker threshold at all. This suggests the possibility that the effect of eserine upon the critical flicker frequency is brought about by enzyme inhibition which prolongs the nicotine-like action of acetylcholine rather than its muscarinelike action. This in turn suggests that, in producing this effect eserine is acting at synapses within the retina. The evidence, unfortunately, is not yet available to be certain that this is the case. It is possible, for example, that the action of eserine on retinal and/or choroidal vessels produced the results obtained and that pilocarpine (though it affected the iris and ciliary muscles just as strongly as eserine) did not penetrate to the retinal and/or choroidal vessels as easily. In order to be certain of this a number of further experiments with other parasympatheticomimetic agents will be necessary.

Granting the possibility of a nicotinelike action of eserine on synapses of nerve cells within the retina, how can it act? It has been suggested that a cholinergic synapse joins the ganglion cells to the bipolar cells and that increased transmission of this synapse could occur when eserine is applied to the eye.[17] In order to account for a decrease in critical flicker frequency in this way it is necessary to assume that the increased synaptic transmission increased the firing of "off" ganglion cells (that is, cells which respond to photic stimulation by a burst of activity when the light is turned off). If this were the case one might expect critical flicker frequency to *decrease* but brightness perception to *increase* after eserine.

In order to check this possibility the following experiment was carried out. In a binocular matching apparatus already described[18] the observer viewed a 2.5-degree by 0.5-degree vertical rectangle slightly above the fixation point with the left eye and a similar rectangle equally far below it with his right eye. These patterns were seen in Maxwellian view with the images of the aperture stops centered in the entrance pupils of the two eyes and smaller than the smallest entrance pupil size. The luminance of the lower rectangle could be varied until it matched the upper in brightness. Prior to the instillation of the one-percent eserine the upper rectangle appeared exactly matched to the lower when the latter had a luminance of seven foot-lamberts. After one drop of eserine to the right eye the lower rectangle soon began to appear dimmer than the upper and it was necessary to raise its luminance to restore the match. When this diminution of the rectangle seen by the left eye had reached its peak (about 24 minutes after the eserine had been applied) the luminance of the rectangle seen by this eye had to be increased to about 45 foot-lamberts in order to match that seen by the normal eye. After this the effect slowly subsided. The general shape of the curve obtained by this binocular matching technique was similar to that obtained in the flicker experiments and it seems quite clear that the results are perfectly analogous. This must mean that topical application of eserine causes a reduction in the brightness of perceived objects (independent of the miosis), that is, eserine has the effect of *inhibition* on visual function.

While it is not possible to be explicit as to how this comes about there is a neuropharmacologic analogy in the firing of ventral horn cells in the spinal cord. The application of eserine will reduce the activity of such a cell. It has been clearly shown in this case that the inhibition is due to an increased firing of a small inhibitory (so-called Renshaw) cell which is excited by the ventral horn cell (by means of a cholinergic synapse). The Renshaw cell in its turn feeds

TABLE 2

EFFECT OF DRUGS ON CRITICAL FLICKER FREQUENCY

Drug	M.A.+ L.E.-R.E.	R.S.J.● R.E.-L.E.	Drug Minus Control		M.A. t	R.S.J. t
			M.A.	R.S.J.		
Control	−0.20	−0.27				
1% Eserine	−1.36	−1.38	−1.16	−1.11	2.40△	3.28□
½% Eserine	−0.95	−1.63	−0.75	−1.36	1.74	3.24□
4% Pilocarpine	−0.19	−0.08	+0.01	+0.19	.04	0.21
1% Strychnine	0.82	+1.20	+1.02	+1.47	3.74○	3.18□

△ $P<0.05$.
□ $P<0.01$.
○ $P<0.001$.
$N=32$.

+ Drug in left eye.
● Drug in right eye.

back onto a ventral horn cell. The synapse of the Renshaw cell on the ventral horn cell itself can be blocked by strychnine and this increases the rate of firing of the ventral horn cell.[19]

Is a like process possible in the retina? A number of experiments designed to answer this question have been carried out. Table 2 illustrates the results of some of them. These experiments were similar to those carried out in Part I. The table illustrates the effects of one-percent strychnine sulfate, eserine (of various concentrations), and pilocarpine on critical flicker frequency. It was found—confirming the data of previous investigators[20]—that one-percent strychnine sulfate applied to the conjunctival sac slightly elevated the critical flicker frequency. This is what might be anticipated if the effects of eserine on flicker discrimination are analogous to its effects on the discharge of the ventral horn cells. If the model is correct it is to be expected that strychnine would block the effects of eserine on flicker discrimination. So far, the attempts to confirm this prediction have proved unsuccessful. Whether this is due to the failure to pace the concentrations and times of instillations of the eserine and strychnine properly or to more fundamental reasons, only further experiments will tell.

It has been reported that application of eserine, nicotine, strychnine, and atropine among other drugs to the isolated frog retina have effects on the electroretinogram which are commensurate with those described on critical flicker frequency in the present paper.[21] Considerable amount of purely physiologic experiments of this sort will be necessary, however, before one will be able to verify the proposed model as a means of explaining the action of eserine on human critical flicker frequency. Such work will also be essential for relating the findings of the present paper to clinical problems of tobacco amblyopia.

SUMMARY

Topical application of one-percent eserine produces a marked decrement in flicker sensitivity in the human eye. The effect appears to be unrelated to any effects of the drug on the iris and ciliary muscles and suggests the possibility of a nicotinelike action of this drug on the retina. Reported effects of other autonomic drugs (regitine, pilocarpine, homatropine, and epinephrine) on flicker discrimination have not been confirmed and it seems likely that previous description of such effects resulted from incomplete control of important experimental variables.

University Hospital.

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Discussion

Paul W. Miles (Saint Louis): I am very glad for the opportunity to discuss this excellent paper. Drs. Alpern and Jampel have shown the way to eliminate important variables. Flicker fusion is such a sensitive and reliable test of vision that it does lend itself well to pharmacologic experiments. When conditions are such that the fusion rate is about 42 per second, the central retest repeatability is plus or minus 0.5 flashes per second. Daily or weekly variation is likewise negligible. Repeatability of flicker fusion on peripheral retina is not as good. The pupil problem bothers me as it does everyone else.

My first slide shows that the flicker rate is related to pupil size in a regular and repeatable fashion. These data were taken with this pupil stick. For any one artificial pupil size, flicker fusion rate remains constant at least two hours.

While it is obvious that an artificial pupil eliminates an important variable, I have not used one clinically. I have found the gain with it is not worth the loss of flicker tests in the periphery. An artificial pupil 1.5 mm. placed 6.0 mm. before the cornea will reduce the fusion rate 30 degrees out from 42 to 10 flashes per second.

When I first began testing visual fields by flicker fusion. I thought I'd have to make the test comparable one patient to another. This seemed to require that each retina tested receive a stimulus of equal intensity and size. I would measure the pupil size by direct comparison with this pupil stick. Then I would refer to a table and select a gray filter to make the retinal stimulus a selected strength. While a natural pupil of 1.5 mm. was often consistent with normal peripheral flicker fusion rates, any gray filter at all, like a small artificial pupil, would reduce the peripheral flicker fusion rates to a useless low level. I concluded that each patient has to be his own reference point in flicker fields—one can only compare different parts of the same field.

Slide 2 shows that we cannot predict central or peripheral flicker fusion rates by the natural pupil size in individuals of any age. Why artificial pupils reduce flicker fusion a fixed amount for two hours at least, but a natural small pupil will apparently not reduce flicker fusion, remains an unsolved problem. Is it possible that an adaptation occurs after several days?

Perhaps an artificial pupil made in a contact lens will become the routine in tests of flicker fields. The peripheral rates should be satisfactory, since the angular extent of the visual field, like that in peeking through a keyhole, depends on the distance from the eye. However, the limiting border would still partly depend on the natural pupil.

When you hold an artificial pupil before an eye, the limiting border will fluctuate or undulate due to spontaneous pupil movements. If you shut the eye a moment and open it, the field has enlarged 10 percent or so, and then slowly constricts again. The effective size of an artificial pupil depends strictly on the distance from the cornea, and the brightness of the retinal image depends critically on this distance.

I have never favored the use of the Broca pupillometer, as used by Alpern and Jampel. First, it is a subjective test. Second, as soon as you put it before the eye, it shuts off the light and causes the pupil being measured to dilate.

I'd like to suggest future studies using the peripheral retina instead of central retina.

Slide 3 shows that previous studies with angioscotomas affected by homatropine or by oxygen are peripheral, not central. If you tested central retina you would miss the effect.

Slide 4 shows the apparent effect on the flicker fusion field of smoking one cigarette. Centrally there was no change in flicker fusion rate, but peripherally it decreased as much as 10 flashes per second. If there were any change in pupil size due to the cigarette, it should affect central and peripheral retina equally.

Slide 5 shows another of several cases I have tested. The depression of the flicker field after smoking one cigarette was very definite, and the recovery from putting a tablet of glyceryl trinitrate under the tongue was equally impressive.

Dr. Louise Lovekin (Fairfield, Connecticut): I have been doing some flicker fusion work this past winter. My approach to the problem of pupil size was simply to measure it in each patient. I have found a definite correlation between the size of the pupil and the height of the flicker fusion, regardless of age and regardless of other things which are affecting the patient. Putting an artificial pupil anywhere between the patient and the flicker, as Dr. Miles said, seemed to introduce another source of error into the system in which there are already a great many sources of error.

I am interested also in the fact that pilocarpine did not have any effect. The literature mentions quite a pronounced effect with it for a short period of time. Dr. Campbell of the Eye Institute in New York has been doing some work on this subject and, I believe, he has found an effect from pilocarpine which lasted for a short period of time. Then, even though the pupil was still contracted, it seemed to wear off, in accord with what Dr. Alpern found with eserine.

I also checked as to whether people did or did not smoke. It was interesting that the people who smoked as much as two packages of cigarettes a day, even in the older age groups, had high flicker rates. There was one woman who was a very heavy smoker, and she had one of the highest flicker rates. It seemed contrary to what one would expect, especially in the periphery of the retina.

Dr. Julia T. Apter (Chicago): On Monday I said I was looking forward eagerly to hearing Dr. Alpern's paper, and I haven't been the least bit disappointed. Perhaps it should be pointed out that there is practically no controversy, that, when strychnine does reach the retina, it does have some effect, as shown by electroretinography. So, if one gets no effect from strychnine locally, perhaps it is not capable of entering through the cornea.

Strychnine is a drug that is not so toxic as one may think. It has been used very widely clinically in very minute doses. In my experiments where I found that it had an effect on the flicker fusion in cats, we used it in doses that were compatible with vital functions in humans—very minute doses. Perhaps one might be venturesome and try systemic administration.

Dr. Mathew Alpern (closing): In regard to using a Broca pupillometer, I agree that this is a crude sort of device. It is rather hard, however, to design a gadget that does exactly what you would like it to do, which is to measure the size of the pupil under the stimulus conditions of the flickering field with the artificial pupil in place. In order to compensate for the effect that Dr. Miles spoke of, we did try to make these measurements of the pupil size under an increased intensity of the light at which the observer was looking so that the net effect of reducing the area of flux going through the pupil was compensated for by the increase in intensity of that light which did get in.

This was only an approximate compensation, however, and I am not prepared to say to what degree we were successful in this respect. However, in general, the important thing to note was that the pupil size which we measured, was always considerably larger than the artificial pupil—the thing we wanted to be certain of. It is quite clear, I think, that any discrepancies or artefacts of pupillometry would not be large enough to account for the effects that we found.

I want to emphasize as strongly as I can the importance of controlling the fluctuating pupil size. I don't think you can do this merely by measuring how large the pupil is.

It is necessary to employ an artificial pupil which will be smaller than the entrance pupil ever can get. This is the only way one can be certain of what variables are involved. I am sure, that the reported effects of pilocarpine on critical flicker frequency are due to this factor.

The matter of how pupil size affects critical flicker frequency is an extremely complex one. It may not be as simple as just varying the amount of light that gets back to the retina. There are intermingled problems of adaptation, and, to my mind, this is all the more reason why one should use extremely small artificial pupils in making measurements of this sort.

In regard to Dr. Apter's comments, there is a clear elevation of critical flicker frequency with strychnine. The thing we were looking for but didn't find was a blocking of the effects of eserine by strychnine. Perhaps the suggestion of stronger concentrations of systemic strychnine may be the answer.

STUDIES ON THE ANATOMY AND PATHOLOGY OF THE PERIPHERAL CORNEA*

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Although extensive studies have been done on the anatomy, physiology, and pathology of the cornea, many of its fascinating mysteries remain unsolved. The studies reported in this paper investigate the peripheral cornea in an attempt to correlate localization of diverse corneal pathology with something other than coincidence. The sparing of a lucid concentric interval at the periphery of the cornea by many cases of hereditary corneal dystrophy, disciform keratitis, and also by cases of measles keratitis in the South African Bantu[1] is curious. The peripheral lucid interval diagnostic of arcus senilis is not completely understood. Finally, the common location of marginal degeneration, the corneal lesions sometimes associated with periarteritis nodosa[2] and the small peripheral ulcers associated with staphylococcal infection, are all very interesting. The combination of these clinical experiences was more than sufficient stimulus to prompt further investigation of the peripheral cornea. These clinical phenomena have previously provoked much discussion and theorization by ophthalmologists. A good example is the paper by Prof. Ernst Fuchs in 1930 entitled "The localization of pathologic changes in the cornea."[3]

I do not intend to summarize the vast amount of literature on the cornea, but merely to relate the facts of this particular study and briefly discuss possible conclusions. The paper can best be divided into anatomic, pathologic and histochemical investigations.

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ANATOMIC STUDIES

MATERIAL

Ten human Eye-Bank eyes which had been fixed in 10-percent formalin were used in this investigation.

METHOD

Two sectors of the cornea were cut out of each eye, using the technique shown in Figure 1. Each sector was triangular in shape with its base in sclera and the point in central cornea. The sectors were imbedded as flat as possible in paraffin (fig. 1-b). The block was cut tangentially. The sections were serially mounted on slides and stained by Masson's trichrome method.[4] The characteristics of the sclera and cornea can best be contrasted by comparing Figures 2 and 4.

The sclera shows bundles of fibers criss-crossing in an irregular fashion. The individual fibers are easily visible in Figure 2. The cornea, on the other hand, appears more homogeneous, and although one gets the impression of a more orderly arrangement than the sclera, the individual fibers are largely obscured.

Between the sclera and the cornea there is an intermediate zone in which characteristics foreign to both cornea and sclera are visible (fig. 3). The general direction of the fibers in this area is circular, as if tending to form a narrow ring of fibers separating the sclera from the cornea.

Figures 5 and 6 emphasize the clarity of the individual fibers in this zone and show the numerous cross communications between fibers, giving a general feathery effect under the high power microscope.

The characteristics of the cornea and sclera were clearly visible in sections from all 10 eyes examined, whereas the intermediate area was distinct in sections from only seven

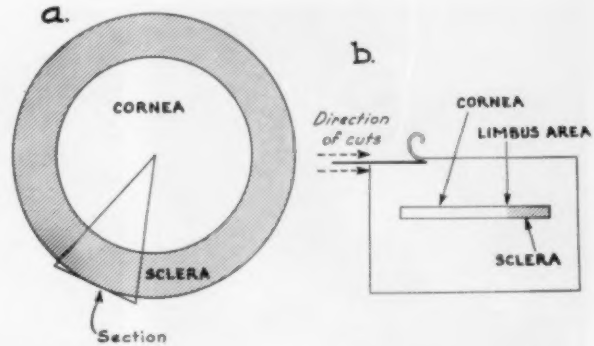

Fig. 1 (Pratt-Johnson), Human eye: Sector for tangential section. (a) The type of sector utilized is shown. (b) The sector is imbedded as flat as possible to facilitate tangential sectioning.

of the 10 eyes examined. The width of the intermediate area when seen was thought to be under 0.50 mm. The intermediate area was seen in sections which passed through the superficial, mid, and deep stroma. More specific data on the intermediate area are not available, as technical difficulties associated with the tangential sections prevented accurate serial examination. An attempt to study the intermediate area in relation to the complete circumference of the peripheral cornea was thwarted by technical difficulties and inconsistencies. I am unable to explain the absence of the intermediate zone in good sections from three of the 10 human eyes examined.

Pathologic studies

A. Human eye-bank material

The microscopical characteristics of arcus senilis were studied in five human Eye-Bank eyes.

Method. The eyes were fixed in 10-percent formalin and sectors were cut as in Figure 1-a. Tangential frozen sections were obtained and stained with oil red-O fat stain.[5]

Results. A typical example of the micro-

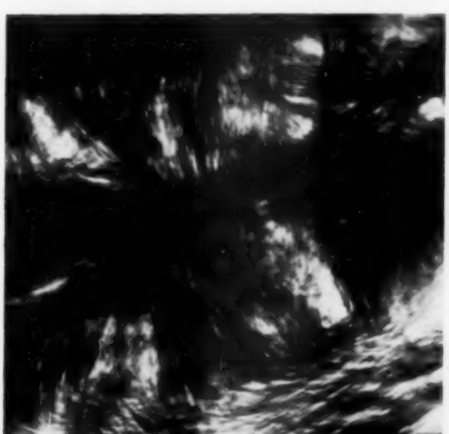

Fig. 2 (Pratt-Johnson). Human eye. High-power tangential section of sclera stained with Masson's trichrome stain.

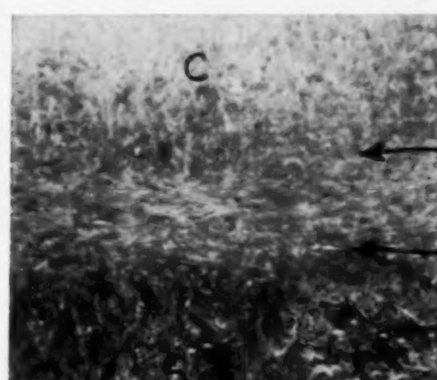

Fig. 3 (Pratt-Johnson). Human eye. Low-power view of tangential section. An intermediate zone of fibers separates the sclera (s) from the cornea (c) (Masson's stain).

Fig. 4 (Pratt-Johnson). Human eye. High-power view of tangential section of the cornea stained with Masson's trichrome stain.

Fig. 6 (Pratt-Johnson). Human eye. High-power view of fibers in the zone intermediate between sclera and cornea (Masson's).

scopical characteristics of these sections is seen in Figure 7. The sclera contains a considerable amount of fat, and the fat responsible for the arcus senilis is seen in the cornea as a band concentric to the sclera but separated from it by a one to two mm. relatively fat-free lucid interval. The arcus is demarcated from the lucid interval peripherally whereas centrally the limits are more vague.

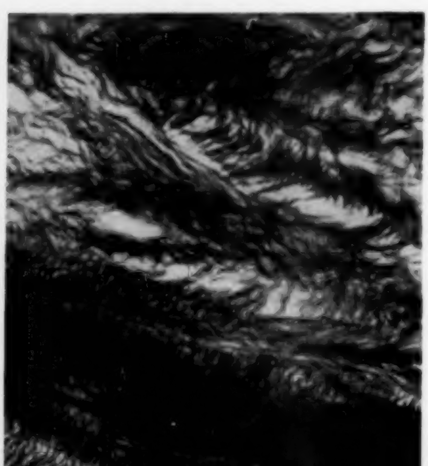

Fig. 5 (Pratt-Johnson). Human eye. High-power view of fibers in the zone intermediate between sclera and cornea (Masson's).

Sections were also examined from eyes from a nine-year-old and 15-year-old donor. These showed less fat in the sclera and no fat in the cornea.

An attempt was made to study the width of the lucid interval with regard to the depth of the stroma. No conclusions were reached and more study is needed to elucidate this point.

B. Experimental study on rabbits

Material. I am indebted to Dr. Meyer Friedman of Mt. Zion Hospital for allowing me to examine his rabbits which he is using for research into atheromatosis and coronary artery diseases, and for his helpful advice in this study. These rabbits are fed on a diet consisting of two-percent cholesterol in three-percent cotton seed oil. After three months, approximately 20 percent show an arcus

Fig. 7 (Pratt-Johnson). Human eye, arcus senilis. Frozen tangential section stained with oil red-O fat stain. Note relatively fat-free lucid zone (l) between the positively staining sclera (s) and the arcus senilis in the cornea (c).

Fig. 8 (Pratt-Johnson). Rabbit eye. Tangential section. Limbal vessels engorged with fat. Some fat seen in surrounding tissue causing round-cell infiltration.

lipoides in the cornea, 80 percent show white deposits of fat in the iris. At this time, the blood cholesterol is approximately 15 to 20 times normal for a rabbit.[8]

Macroscopic results. The localization of fat deposits in the rabbit cornea under these conditions of "fat intoxication" varies and in only half the rabbits was a lucid interval similar to human arcus lipoides developed. In some of the rabbits with macroscopic fat deposits in the cornea, an inflammatory response with limbal neovascularization and congestion was seen.

Histologic results

Method. Six eyes showing macroscopic arcus were enucleated and fixed in 10-percent formalin. Tangential frozen sections were obtained on sectors cut as in Figure 1-a to pass through the area containing the arcus. Figure 8 shows the limbal vessels engorged with fat. In Figure 8, some of the fat has escaped from the blood vessels and begun to migrate into the cornea. It is associated with an inflammatory response and round-cell infiltration. This eye did not exhibit a lucid interval. Figure 9 shows the tendency of the fat in the cornea to collect in the paralimbal area after traversing the periph-

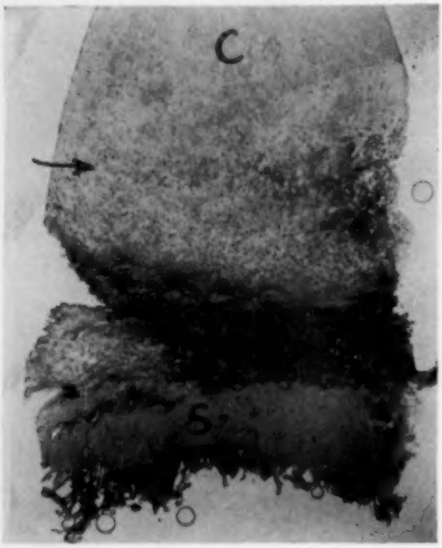

Fig. 9 (Pratt-Johnson). Rabbit eye, experimental arcus lipoides. Arrow indicates area in which fat is tending to accumulate after migrating from limbal vessels.

eral zone. This eye macroscopically did exhibit a lucid interval.

Histochemical studies

Toluidine blue, a stain which is known clearly to differentiate the sclera from the cornea because of the histochemical differences occasioned by the acid mucopolysaccharide in the cornea, was chosen.

Material. Four human Eye-Bank eyes were used after fixation in 10-percent formalin.

Method. A sector from each eye was cut as in Figure 1-a. A small cut with a sharp razor edge was made at the point where the opaque sclera became clear cornea. Tangential frozen sections were then cut. The sections were placed onto slides and divided into two groups. One group was stained with oil red-O fat stain. Those in the other group were placed in eight-percent basic lead acetate and formalin mixture and left to soak for 24 hours. They were then stained with a 0.1-percent toluidine blue in 1.0-percent alcohol.[6] (Since it has been suggested that the efficacy of the stain improves on aging, it should be mentioned that the stain used was in fact two years old!)

Results. 1. Sections stained with oil-red-O. Sections from all four eyes showed fat staining characteristics of arcus senilis (fig. 10).

2. Sections stained with toluidine blue. In all sections from the four eyes, the sclera was clearly demarcated from the cornea, the sclera staining blue green and the cornea navy blue. In two of the eyes, the stain differentiated two zones in the cornea, a narrow pale purple peripheral zone and the navy blue zone in the center (figs. 11 and 12). Prior to imbedding these sections, an incision with a razor blade was made at the edge of the sector at the junction of the clear cornea with the sclera. The pale purple peripheral zone lay central to the cut and therefore was in clear cornea.

It is interesting to compare Figure 10 with Figures 11 and 12. The lucid interval of arcus senilis would seem to correspond

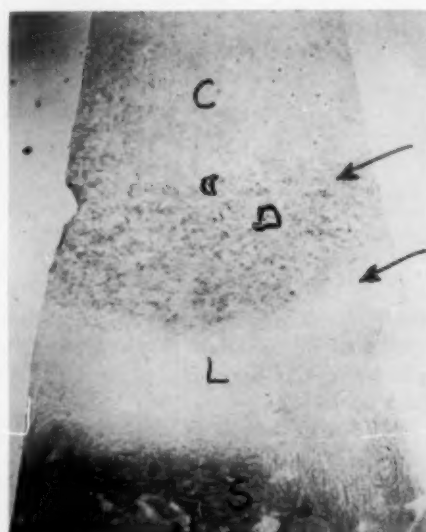

Fig. 10 (Pratt-Johnson). Human eye, arcus senilis). Tangential frozen section stained with oil red-O fat stain. The fat free lucid interval (L) is clearly seen between the fat in the sclera (s) and the fat of the arcus in the cornea (c).

roughly with the peripheral corneal zone demarcated by toluidine blue.

I am unable to explain specifically why two zones of the clear cornea are differentiated by this stain, but suggest it is evidence of some different histochemical structure, possibly accentuated by metabolic changes associated with the aging process and limbal vascular sclerosis. I should point out, however, that in subsequent work I have found the two corneal zones differentiated by this technique in the eye of a 26-year-old woman who had no signs of arcus or premature aging or vascular disease.

Summary of findings

1. There is a histologically distinct zone between the corneal stroma and sclera. It has been seen in tangential sections passing through the superficial, mid, and deep stroma of seven out of 10 human eyes examined. It is estimated to be less than 0.50 mm. in width.

2. Toluidine blue stain has differentiated two corneal zones on tangential frozen sections of two out of four human Eye-Bank eyes examined. The small peripheral zone corresponds to the lucid interval of arcus senilis and is approximately one to two mm. wide.

3. Experimentally produced arcus in rabbits sometimes exhibits the lucid interval between the corneal arcus and the limbus similar to that seen in arcus senilis in humans.

Fig. 12 (Pratt-Johnson). Human eye. Tangential frozen section stained with toluidine blue. Arrow (X) points to the razor cut incised before sectioning at the macroscopical junction of opaque sclera with clear cornea. Hence the peripheral corneal zone (between arrow A and P) is clear cornea.

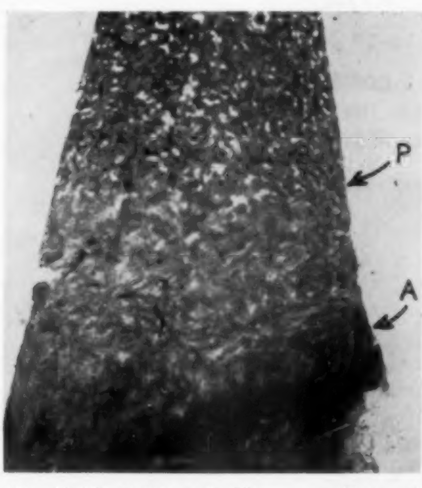

Fig. 11 (Pratt-Johnson). Human eye. Tangential frozen section stained with toluidine blue. The arrow (A) differentiates the sclera from the peripheral corneal zone. The arrow (P) designates the area of the paralimbal ring.

Discussion, impressions, and theories

Although I am unaware of any published work in support of a histologically distinct area situated between the sclera and cornea, one should recall the work of Kokott, who demonstrated circular fibers at the periphery in the superficial and deep stroma by a method of interstitial injection.[7]

Discussion

The anatomic studies warrant in the present stage of the investigation only the statement that sections from seven out of 10 human Eye-Bank eyes revealed a roughly concentric narrow band of fibers interposed be-

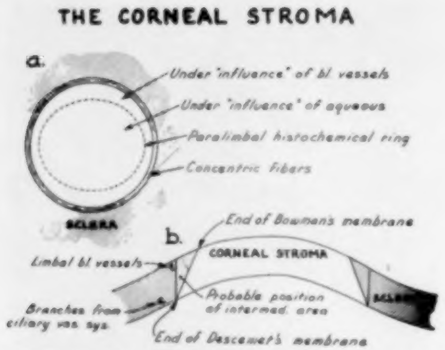

Fig. 13 (Pratt-Johnson). Diagrammatic representation of hypothesis that the corneal stroma of the human eye consists of two zones.

tween the cornea and sclera.

The histochemical and pathologic studies are best considered together. It was interesting to note in the two eyes in which toluidine blue differentiated a central and peripheral zone, that the peripheral zone corresponded closely to the lucid interval shown between the fat staining material in the cornea and the sclera seen in the section stained with oil red-O. The fatty material appeared concentrated in the junctional area of the central and peripheral zones. These facts have led me to postulate a theory that the stroma consists of two areas which differ metabolically and histochemically.

Hypothesis of two stromal zones

This hypothesis postulates that the corneal stroma consists of two zones. There is a larger central and smaller concentric peripheral zone in the clear cornea. The junction of the two areas is referred to, for descriptive purposes, as the paralimbal ring. It is suggested that the stroma peripheral to the paralimbal ring is oxygenated from the limbal vessels, whereas the central stroma obtains its oxygen from elsewhere, possibly the aqueous. The paralimbal ring may also represent a histochemical barrier.

Clinical evidence in support of this hypothesis (fig. 14)

1. Hereditary corneal dystrophy, measles keratitis (South Africa) and disciform kera-

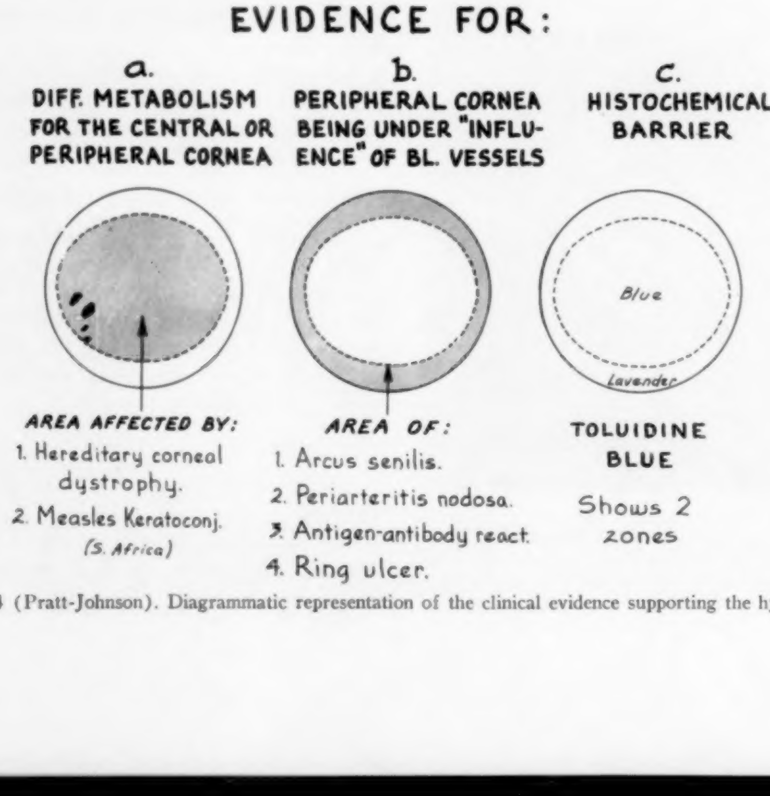

Fig. 14 (Pratt-Johnson). Diagrammatic representation of the clinical evidence supporting the hypothesis.

titis are not associated with vascular disease, or the aging process. Therefore these conditions do not affect the peripheral zone.

2. Lesions associated with vascular sclerosis or the aging process would be particularly liable to affect the paralimbal area first as this is the region of the peripheral area farthest from the limbal vessels. The localization of arcus similis and marginal degeneration is thus explained.

3. If vascular sclerosis is severe and progressive one would expect degeneration in the cornea to spread from the paralimbal ring through the peripheral corneal zone. The corneal lesions associated with periarteritis nodosa can produce this picture.

4. If antigen is injected in small quantity in the center of the systemically sensitized rabbit, the reaction caused by antigen-antibody union localizes to the paralimbal ring area.[10] This lends support to this hypothesis, since the antibody from the serum in the limbal blood vessels seems to reach the peripheral but not the central corneal zone.

5. The pathogenesis of the classical ring ulcer also seems to support the hypothesis.

Pathogenesis of arcus senilis and arcus juvenilis

a. Arcus senilis (fig. 15-b)

Arcus senilis is depicted as a fatty degeneration in the paralimbal ring area due to mild limbal vascular sclerosis associated with the aging process. According to this hypothesis the localization is logical since it is situated in the most central part of the peripheral area, that is, the region most distal from the limbal blood vessels. Clinical evidence is readily available to support this interpretation. It has been shown that fatty changes in the paralimbal area are demonstrable microscopically in 100 percent of people over the age of 50 years, most of whom have normal blood lipids.[9a] This is, of course, good evidence that the change in arcus senilis is predominantly an aging change.

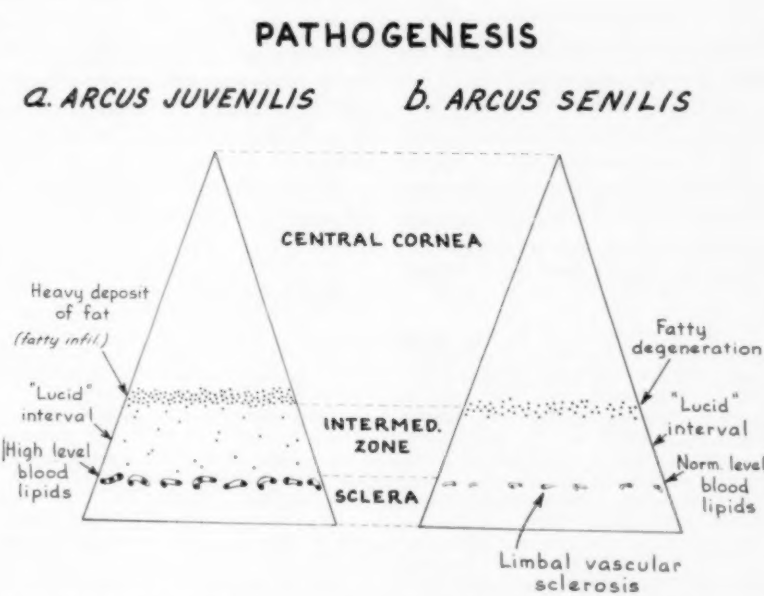

Fig. 15 (Pratt-Johnson). Diagrammatic representation of the pathogenesis of arcus juvenilis and arcus senilis in humans.

b. Arcus juvenilis

This would appear to be of two types:

1. In early onset of the aging process, sometimes associated with premature graying of the hair and an hereditary trait.

2. A fatty infiltration of the paralimbal area of the cornea associated with high blood lipids. It may be associated with xanthomatosis, lipoid nephrosis, and other diseases associated with abnormally high blood lipids.

According to this hypothesis, lipid would overflow from the limbal vessels, pass into the cornea, precipitate, and accumulate in the paralimbal ring zone where there is a histochemical change. This would explain the lucid interval seen in arcus juvenilis and correlate it with the high blood lipids found in the majority of cases.[9b]

c. Arcus lipoides in rabbits

The mechanism would seem similar to arcus juvenilis associated with high blood lipids.

Summary

This study attempts to investigate the localization of some corneal pathology. For example, hereditary corneal dystrophy, disciform keratitis, and measles keratitis (South Africa are limited to the central cornea, whereas arcus senilis, marginal degeneration, and the corneal lesions of periarteritis nodosa characteristically affect the periphery.

The study is divided into three experiments:

1. *The anatomic studies* using tangential sections and Masson's trichrome stain showed a narrow band of fibers concentric to the limbus, interposed between cornea and sclera, in sections from seven out of 10 human Eye-Bank eyes examined.

2. *Pathologic studies.* The pathogenesis of arcus senilis and experimental arcus in rabbits was studied using tangential frozen sections and oil red-O fat stain. Evidences put forward show that arcus senilis results from fatty degeneration. On the other hand arcus juvenilis may be of two types. (1) premature arcus senilis, (2) as a result of fatty infiltration.

3. *Histochemical studies.* These utilized toluidine blue stain of frozen sections from human Eye-Bank eyes. In two out of four eyes this stain differentiated the cornea into a larger central and smaller peripheral zone. It is interesting and significant that the lucid interval of arcus senilis corresponds to the peripheral corneal area.

On these findings a hypothesis is presented in which the corneal stroma is divided into two zones, a larger central and smaller peripheral zone. Evidence is given to show that these two areas may differ histochemically and metabolically. This hypothesis may help in explaining the localization of some corneal pathology. It seems likely that paralimbal pathology may be the corneal manifestation of some systemic diseases.

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Discussion

A. Ray Irvine, Jr. (Los Angeles): For some years we have been studying tangential sections of the trabeculae and limbal region in an effort to determine the nature of trabecular sclerosis and its relationship to glaucoma. Our studies have been concerned with the changes in elastic tissue and endothelial cells of the trabeculae and neighboring structures. In doing these studies, we encountered technical difficulties, both in inconsistency of staining reaction and the inability to consistently section in a true tangential plane.

In a recent review of our material, I was unable to find a definite intermediate zone between cornea and sclera that corresponded with the circular fibers described by the author. However, I freely admit that his photographs indicate that his sectioning was better than ours and that our technique of dissolving celloidin from old blocks, remounting and sectioning in paraffin may have produced sufficient artefact to obscure the fine changes he describes.

One of the principal differences between the cornea and the sclera is the presence of elastic tissue in the latter. We found the elastic tissue concentration to be greatest in the trabeculae and adjacent sclera. It is of interest that in this area in meridional sections, many of the scleral bundles appear to travel in a circular manner and to be separated from one another by elastic tissue threads. This rather distinctive arrangement disappears at the midstromal level. Because we are dealing with tangential sections of the wall of a sphere, the central portion of the section will be somewhat deeper than the peripheral parts. Therefore, in deep sections, between the trabeculae and the midscleral region, the circular bundles might appear to be interposed between cornea and radially directed scleral fibers. A similar effect could be achieved in superficial sections cut somewhat obliquely but should not occur in superficial sections cut truly tangentially. It would be interesting to know if the circular fibers described by the author contain elastic tissue.

In our experience, the metachromatic staining of acid polysaccharides has given rise to inconsistencies that make interpretation of results in a small number of cases difficult. Toluidin blue has long been known to have metachromatic properties. Unfortunately the absorption spectra and therefore the color and shade of this dye is affected by factors other than the addition of chromotropic substances. Some of these other factors are concentration of the dye, the temperature, pH, and the presence of alcohol in the tissue. The physical chemical nature of metachromasia is not completely understood to my knowledge and the presence of a physiologic barrier cannot be presumed to exist on the basis of a slightly different staining reaction in two out of four eyes.

I have enjoyed reading the author's paper very much and commend him upon his method of investigating this type of problem. As he has mentioned, considerably more work of similar kind will have to be done before his hypothesis will be proved or disproved.

Dr. Lorenz E. Zimmerman (Washington, D.C.): If I understood Dr. Pratt-Johnson correctly, the method that he had to use to demonstrate the metachromatic zone in the peripheral cornea was tangential sectioning, and this was true in only seven of 10 specimens, and he could not account for the absence of the metachromatic zone in the peripheral cornea in the other three cases.

If my understanding is correct that it requires tangential sectioning, this must be a zone that does not extend through the full thickness of the cornea. It must be a stratified area which in conventional meridian sections would be so thin that it would be difficult to see, but by tangential sectioning it would be possible to catch it.

I would like first to have that point clarified. Is this just a level? If it is just a level, is it close to the surface or is it down deep in the cornea close to the endothelium?

Along with that question, how does this correlate with the zones of lipid deposition in arcus senilis? Do you find the level the same in the two situations?

With regard to the other clinical correlation or experimental correlation, this zone of antigen-antibody precipitation that you get, here again I would like to ask a question because in the pictures the author showed, and in the paper that was presented earlier this week, the zone of antigen-antibody precipitation seems to move.

In the picture that you showed the ring seems to come out all the way to the limbus in some areas. What happens to the zone in that area, particularly since this zone moves?

Also, in similar experiments if you have an animal that is not sensitized, and if you inject antigen in one part of the cornea and antibody in the other part, it is my understanding that the cornea acts merely as an agar diffusion medium, and you get the zones of precipitation almost anywhere in the cornea, depending upon where antigen and antibody are interacting. How does this correlate with this metachromatic zone, or how can you use the metachromatic zone at the periphery to account for a central zone of precipitation?

Dr. Albert M. Potts (Cleveland, Ohio): It is probably worth making one precautionary statement in regard to drawing conclusions from this very excellent piece of histological work and work done with large molecules such as proteins, and conclusions drawn from the behavior of fat soluble substances, and carrying this over to a generalized biological barrier.

It has been known since the work of Gruber in the past century that small molecules diffuse from the limbus quite close to the corneal center. This type of thing was repeated by us with diffuse radioactive isotopes of ions some years ago.

DR. JOHN A. PRATT-JOHNSON (closing): I would like to thank Dr. Irvine for the trouble he has taken to analyze the paper, and for his splendid remarks. In reply, there is some evience to support the idea of there being concentric fibers interposed between the sclera and cornea; this evidence is in the literature. Dr. Kokott did some very careful work in Germany with interstitial injections and demonstrated concentric fibers in mid and in deep zones of the cornea.

In my anatomic study, there were 10 human Eye-Bank eyes that were examined for concentric circular fibers. Good sections only were taken. If an eye was sectioned and the sections were not good, that eye was not examined. As Dr. Irvine pointed out, tangential sectioning is extremely difficult to do. In 10 eyes in which excellent sections were obtained, we were able to demonstrate this area in only seven. I am unable to explain this discrepancy. In the seven positive eyes, circular fibers were demonstrable in superficial and in deep sections.

The clear differentiation of the central and peripheral corneal zones by toluidine blue must be due to some histochemical difference. The word histochemical barrier is coined for want of a better word to avoid verbosity in describing the junctional area. The justification of the encorporation of the word barrier rests with the interpretation of the hypothesis presented.

In reply to Dr. Zimmerman: Yes the antigen-antibody reaction in the cornea is similar to the agar plate phenomenon and one can certainly get a line of precipitation in the middle of the cornea if one injects antigen on one side and antibody on the other. In some of our experiments actively immunized animals which had been immunized for months were used. Despite this fact, significant penetration of the circulating antibody was confined to the peripheral zone of the cornea. After injection of antigen into the middle of the central zone, diffusion of the antigen slowly began toward the peripheral zone. It reached this area in about 20 hours and, as expected, formed a line of antigen-antibody precipitate in the junctional zone. This is a consistent and quite repeatable finding and is very much in favor of this hypothesis.

I will have to take up the other very interesting points raised with the discussers personally at another time.

ACID MUCOPOLYSACCHARIDE IN THE RETINAL PIGMENT EPITHELIUM AND VISUAL CELL LAYER OF THE DEVELOPING MOUSE EYE*

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Recent histochemical studies[1-5] have revealed the existence of a mucoid material between the sensory retina and the pigment epithelium. Particularly abundant in man, it is readily preserved by ordinary fixatives, and easily stained by alcian blue[6] (color plate A and C) or by the Rinehart-Abul-Haj[7] modification of the Hale[8] procedure (color plate B). The precise chemical nature of this mucoid material has not been determined but it appears to be an acid mucopolysaccharide which is resistant to both streptococcal and bovine testicular hyaluronidase. Although the presence of a kind of ground substance between the visual cells and the pigment epithelium has long been suspected, it has escaped recognition because it is not stained by ordinary dyes. Even the periodic acid-Schiff procedure fails to demonstrate this substance just as it fails to demonstrate hyaluronic acid.

Upon examination of the retina with low magnification it is readily apparent that only the zone outside of the external limiting membrane reacts positively to stains for acid mucopolysaccharides. With greater magnification it becomes clear that the mucoid material is a sort of ground substance in which the visual cells are embedded. The purpose of this substance is unknown, but several suggestions as to its function can be made. It might be designed to facilitate exchange of essential materials between the choriocapillaris and the visual cells. It might provide the ideal optical environment for rod and cone function. It might be sticky and promote adherence of the sensory retina to the pigment epithelium.

One observation often made in the course

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of preparing normal human eyes for histologic study has a direct bearing on the last of these suggestions; that is, it is hard to keep the retina in place against the pigment epithelium. If the outer surface of the retina, detached during preparation by manipulation, is examined carefully, many pigment granules can usually be seen clinging to the tips of the visual cells. This suggests the presence of a sticky substance. This thin layer of adherent pigment granules is not observed on retinas which become detached in vivo.

The source of this ground substance is obscure. Several observations made on human material suggest that it may be elaborated by the sensory cells themselves. In sections from several retinoblastomas containing well-differentiated Flexner-Wintersteiner rosettes stained for acid mucopolysaccharides, a thin film of hyaluronidase-resistant mucoid has been noted within the lumen of the rosettes (color plate D).[4] A similar mucoid substance which resists hyaluronidase has been observed in some of the non-neoplastic rosettes of retinal dysplasia. Furthermore, in the human eye and the eyes of other pigmented species, the retinal pigment epithelium does not appear to contain any stainable acid mucopolysaccharide.

The studies to be reported herein were conceived in an effort to provide further evidence that the visual cells themselves secrete the ground substance in which their distal processes are embedded. We decided to study the mouse eye from birth, when the visual cells have not yet begun to differentiate, until 21 days after birth, when the retina is fully differentiated. The mouse eye was selected, not only because its retina is incompletely differentiated at birth (fig. 1) but also because of the availability of strains of mice in which the eye, though seemingly normal in development for the first 10 to 12 days of postnatal life, then shows a strikingly precipitous and selective degeneration of the partially differentiated rods and their nuclei (fig. 2).[9-14] These stains were available in both pigmented and albino stock.

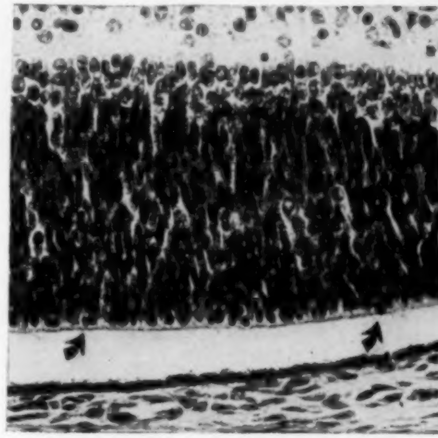

Fig. 1 (Zimmerman and Eastham). The newborn mouse retina is only partially differentiated. Numerous mitotic figures are present in the outermost layer of nuclei (arrows), and the rods have not yet begun to differentiate. (AFIP Neg. No. 57-17996. Hematoxylin-eosin, ×380.)

Our reasoning was as follows:

If the rods produce the interstitial substance, then a study of the differentiating retina might be expected to reveal a temporal and spatial relationship of the acid mucopolysaccharide to the outer layer of retinal cells. Furthermore, it might be anticipated that the acid mucopolysaccharide would be absent in the rodless retina. The albino strains (in which the "pigment epithelium" contains no pigment) were included in order to provide more conclusive evidence that the retinal pigment epithelium is not the source ot this mucoid substance.

At the time these studies were initiated we had not yet learned of Sidman's recent work.[2] Utilizing a modified freeze-substitution method of fixation in order to obtain better preservation of histologic detail and to conserve the chemical constituents of the tissues, Sidman observed that the interstitial zone between the layer of epithelium and the visual cells of the mature albino mouse eye contains at least three components: (1) minute periodic acid-Schiff-positive, alcian blue-negative granules contained in the apical cytoplasm of the pigment cells; (2) thin

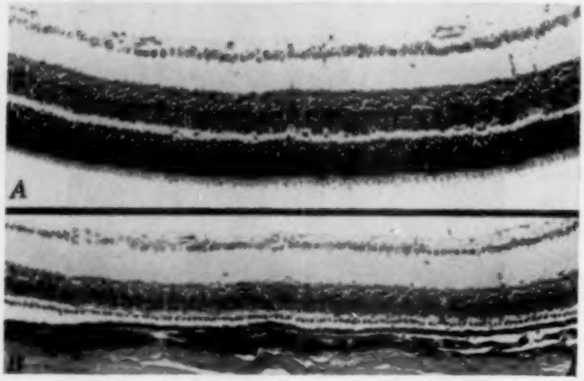

Fig. 2 (Zimmerman and Eastham). Retinal differentiation of "rodless" mice proceeds normally until after the 10th to 12th day; then the outer nuclear layer rapidly disintegrates and visual cells fail to differentiate. (A) Rodless C3H retina at 11 days appears essentially normal; rod inner segments have partially differentiated. (B) Rodless C3H retina at 21 days has an outer nuclear layer which is reduced to a single row of nuclei and no inner or outer segments of the rods remain. (AFIP Neg. No. 57-18057. Hematoxylin-eosin, ×115.)

PAS-positive fibrils of irregular shape which project from the free surface of the pigment cells toward the rods and which are coated with an alcian blue-positive mucoid; and (3) homogeneous alcian blue-positive material in the space between the pigment cell layer and the rod outer segments. Since Sidman apparently studied only the completely differentiated retina, he was not able to make any observations as to the site where the alcian blue-positive matrix first appeared during development.

Materials and Methods

Pigmented and albino strains of normal and rodless mice were studied from birth to 21 days. Breeding pairs of the pigmented C3H rodless mouse, first described by Dunn[12] and subseqently studied in much greater detail by Noell,[14] were obtained through the courtesy of Dr. Walter E. Heston of the National Cancer Institute, National Institutes of Health, Bethesda, Maryland. Two litters of newborn SWR rodless Swiss mice, an albino strain that has been inbred for about 60 generations, were kindly furnished by Dr. Margaret K. Deringer, also of the National Cancer Institute. For controls we used Bagg-Swiss albino mice and pigmented hybrid mice bred by mating rodless C3H males with Bagg-Swiss albino females. These mice were killed at birth and at successive stages of

Color plate (Zimmerman and Eastham). Acid mucopolysaccharide in the retinal pigment epithelium and visual cell layer of the developing mouse eye.

A. Normal adult human retina stained with alcian blue and counterstained with nuclear fast red. The visual cells, stained by the nuclear fast red, are coated with a mucoid interstitial substance stained by the alcian blue. Observe that pigment granules cling to the tips of the visual cells. (AFIP Neg. No. 57-13640. X700.)

B. Normal adult human retina stained by the Rinehart-Abul-Haj colloidal iron technique; Van Gieson counterstain. The mucoid ground substance in which the visual cells are embedded is again revealed as a deep blue-staining matrix. (AFIP Neg. No. 57-13915. X230.)

C. Normal adult human retina stained by the combined alcian blue-periodic acid-Schiff method. The rod outer segments (lower half of field) are recognized by their vivid magenta color. Between them is an abundant bright blue mucoid matrix. A layer of pigment granules clings to the tips of the visual cells. (AFIP Neg. No. 57-13994. X720.)

D. Retinoblastoma stained with alcian blue. The neoplastic retinal cells have differentiated to the point of forming rosettes lined by abortive visual cells. Within the lumen of these rosettes is a mucoid substance stained by alcian blue, similar in appearance to that shown in A and C. (AFIP Neg. No. 57-14083. X540.)

E. Retinal pigment epithelium of a 10-day-old C3H mouse. Myriad delicate fibrillary processes stained with alcian blue project inward from the pigment epithelium. (AFIP Neg. No. 58-7603. X115.)

F. Retina and retinal pigment epithelium of a 17-day-old C3H mouse stained with alcian blue. The outer nuclear layer is reduced to a row of pyknotic nuclei and the visual cells have degenerated. Strands of alcian blue-positive material bridge the gap between the pigment epithelium and the outer nuclear layer. (AFIP Neg. 58-7594. X630.)

G. Retina and retinal pigment epithelium of a nine-day-old Bagg-Swiss albino mouse stained with alcian blue. A zone of concentrated alcian blue-positive intracytoplasmic particles is present along the inner border of the epithelial cells. Less mucoid material is present about the visual cells, which at this stage are very incompletely differentiated. (AFIP Neg. No. 58-7596. X630.)

H. Ciliary body and root of iris of 21-day-old albino mouse stained with alcian blue. There is a selective staining of the outer layer of epithelium which, in pigmented species, would be densely packed with pigment granules. The inner layer of epithelium shows a negligible uptake of the dye. (AFIP Neg. No. 57-17759. X265.)

I. Ciliary body and root of iris of 13-day-old C3H mouse, bleached and then stained with alcian blue. A similarly stained but unbleached adjacent section from the same block is shown in J. The alcian blue-positive intracytoplasmic material has a distribution which is identical to that of the melanin pigment. This is true of the uveal melanocytes as well as of the pigment epithelium. (AFIP Neg. No. 58-8210. X265.)

J. Ciliary body and root of iris of 13-day-old C3H mouse; alcian blue stain of unbleached section adjacent to that shown in I. (AFIP Neg. No. 58-8211. X265.)

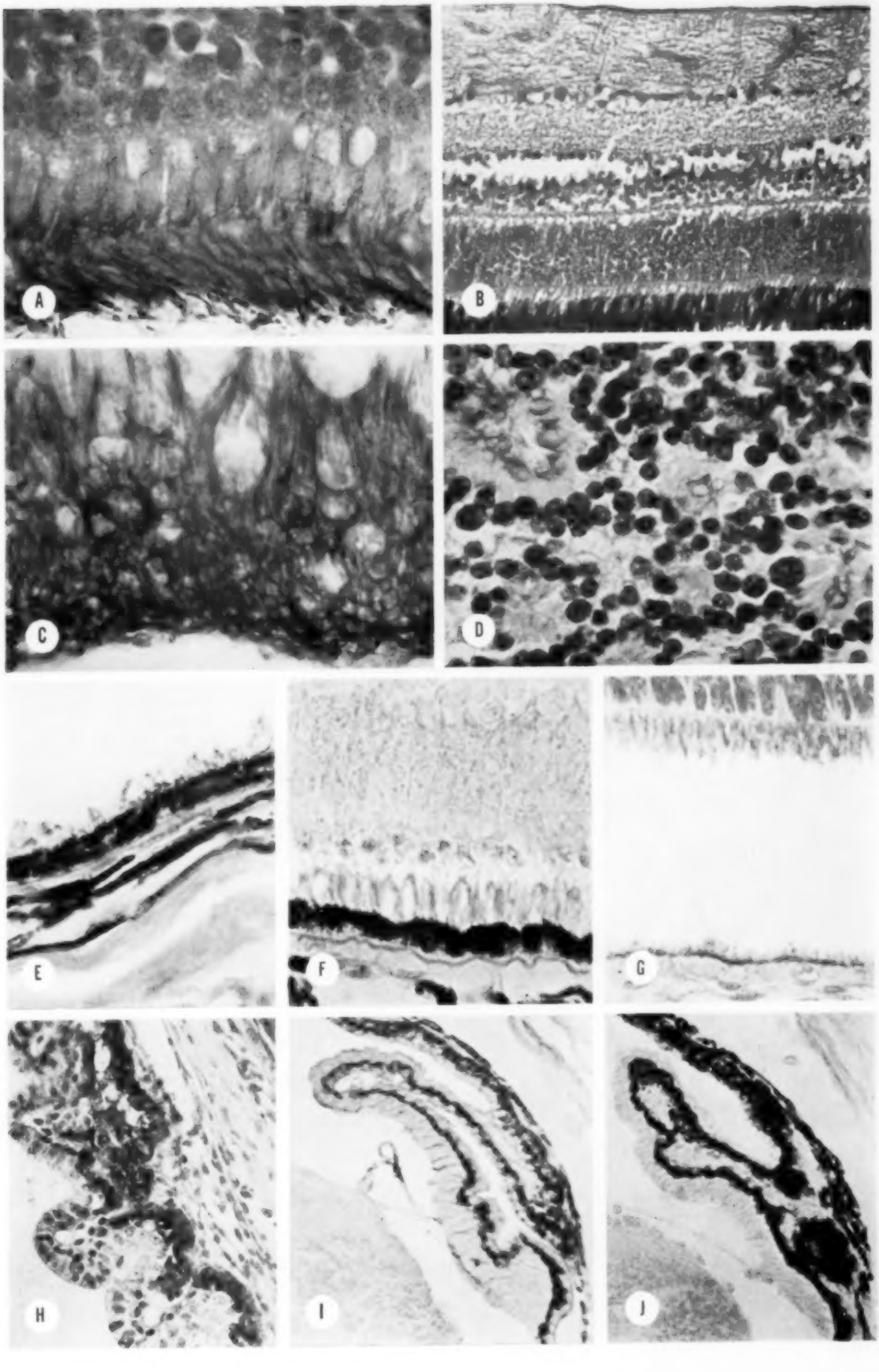
A
B
C
D
E
F
G
H
I
J

Fig. 3 (Zimmerman and Eastham). One-day-old albino mouse eye sectioned in situ after Bouin fixation and paraffin embedding. (AFIP Neg. No. 58-7595. Alcian blue with nuclear fast red. ×80.)

development to full maturity. The C3H and Bagg-Swiss albinos were killed daily from birth to 21 days. The mice were decapitated, and the jaw and the fur removed. The heads were fixed in Bouin's solution for 24 to 48 hours, embedded in paraffin, and sectioned at 7.0μ in the horizontal plane. At about seven days after birth calcification of the cranial bones required treatment in five-percent solution of nitric acid in 10-percent formalin for one to four days. With this technique the eyes were sectioned in situ. At least one and usually both eyes of each specimen contained the optic nerve and pupil in the plane of section (fig. 3).

Serial sections made through the horizontal meridians were stained with hematoxylin and eosin, alcian blue, colloidal iron, and by the periodic acid-Schiff method (PAS). Although the results obtained with alcian blue and with the Rinehart-Abul-Haj technique tend to be parallel, alcian blue is more reliable, more specific, and generally easier to use. Combination of alcian blue and PAS were also employed. The effects of diastase and of bovine testicular and streptococcal hyaluronidase on the staining reaction of acid mucopolysaccharides were tested periodically from birth to 21 days. The bovine enzyme used was Wyeths' Wydase (150 TRU/ml.) while the streptococcal hyaluronidase (0.5 to 1.0 mg./ml.) was obtained through the courtesy of Dr. Emily Emmart of the National Institutes of Health. Dr. Emmart, who prepared this enzyme, found it to have a potency of 996 TRU/mg. Sections prepared from the pigmented strains were also stained for acid mucopolysaccharide after they were bleached with 0.25-percent potassium permanganate for 30 minutes.

In addition to the rodless SWR Swiss albino mice provided by Dr. Deringer, we have been able to study material from rodless albino mice from two other sources. Through the courtesy of Dr. Norman Ashton, we obtained blocks from some of the Swiss albino mouse eyes previously studied and described by Tansley.[10,11] Additional

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rodless albinos were discovered in our own colony of Bagg-Swiss albino mice which were being used as controls. We have made no effort to determine the incidence of rodless retinas in this colony, but Noell found electroretinographic and histologic evidence of this hereditary condition in 18 percent of the animals in his colony of Swiss albino mice.[14]

Results

General distribution of acid mucopolysaccharides

In general, the amount and distribution of stainable mucopolysaccharide in the nonneural tissues of the developing mouse eye seemd to be similar in albino and pigmented strains. The faint diffuse alcian blue-staining* of the corneal stroma seen at birth increased during the first week. Virtually no staining was observed in the sclera except posteriorly about the optic nerve. The cornea and sclera were PAS-positive. The vitreous body at birth did not stain with alcian blue; neither did the sensory retina or optic nerve. The vitreous was moderately PAS-positive as was the internal limiting membrane of the retina. A very delicate film of alcian blue- and PAS-positive mucopolysaccharide was present along the inner surface of the cells in the region of the ora serrata and pars plana. The faint staining reaction of the cornea and of the film on the inner surface of the ciliary epithelium was only slightly lessened by treatment with bovine or streptococcal hyaluronidase for two hours and was not changed by treatment with diastase. On about the ninth day of life alcian blue revealed diffuse staining of the vitreous.

Retinal acid mucopolysaccharide in pigmented mice

Until about nine days after birth, alcian-blue positivity was not observed in the sensory retina, and examination of the pigment epithelium also failed to reveal any blue material. Then delicate blue fibrillary processes were first seen projecting inward from the free surface of the pigment epithelium (color plate E). These were also weakly PAS-positive. A very thin zone of alcian blue-positive precipitate could also be observed about the free (outer) tips of the developing visual cells. The staining intensity of this interstitial mucoid material gradually increased, although it never reached the degree observed in the human eye.

In the C3H mice the earliest evidence of retinal degeneration was noted between the 11th and 13th days, and by the 15th day a remarkable depletion of nuclei in the outer nuclear layer had taken place. Changes in the stainable mucoid interstitial substance, however, lagged behind those in the sensory retina. By the 17th day the outer nuclear layer was reduced to a single row of cells and the cell bodies were almost completely degenerated, but faint staining with alcian blue persisted (color plate F). Because of the heavy pigmentation of the pigment epithelium in the C3H mice, it was not possible to detect changes within the cytoplasm of these cells.

Retinal acid mucopolysaccharide in normal albino mice

Examination of the eyes of albino mice stained for mucopolysaccharides led to a most unexpected observation. At birth, the staining reaction of the neuroepithelial tissues derived from the outer layer of the optic cup differed strikingly from that of tissues derived from the inner layer of the optic cup. The partially differentiated sensory retina of the newborn mouse did not react positively with alcian blue or colloidal iron, and only the delicate lamellae along the internal limiting membrane gave a positive PAS reaction. Likewise, in the ciliary body and iris, only a thin film of mucoid secretion which coated the inner surface of the innermost layer was stained by alcian blue and by the PAS reaction. Cells of the outer epithelial layer, how-

* Unless a specific statement is made to the contrary, it may hereafter be understood that results obtained with the Rinehart-Abul-Haj technique were similar to those with alcian blue.

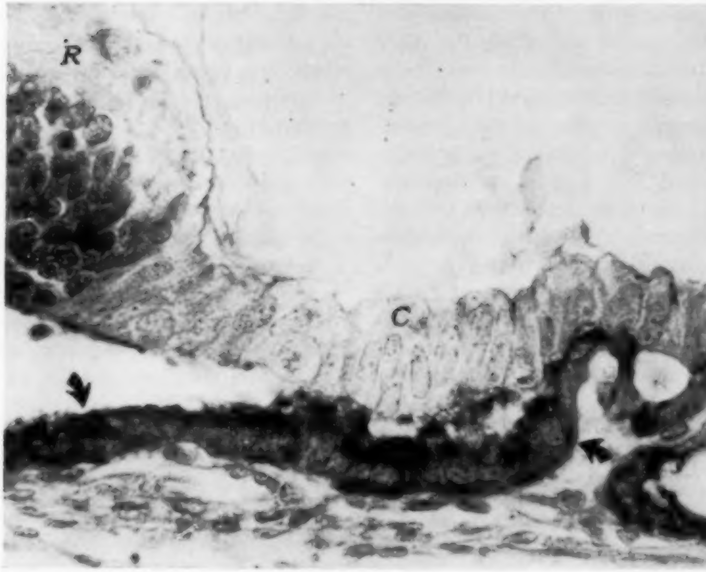

Fig. 4 (Zimmerman and Eastham). In the albino mouse, there is an intense and selective staining of the outer neuro-epithelial layers (arrows) by alcian blue, while the sensory retina (R) and the inner layer of ciliary epithelium (C) are not stained. (Alcian blue with nuclear fast red. AFIP Neg. No. 58-7598. ×600.)

ever, were stained intensely by both methods. With alcian blue, minute bright blue particles were seen throughout the cytoplasm of these cells, while with the PAS reaction vivid red particles were visualized. With both staining methods, the number of intensely stained cytoplasmic particles was greatest in the ciliary body (fig. 4 and color plate H), diminishing toward the pupil and toward the retina. Nevertheless, it was apparent that all of the cells throughout the retinal epithelium contained these particles. With oil-immersion microscopy they appeared to be filamentous. At birth these particles seemed to be rather evenly distributed in the flattened epithelial cells.

When the alcian blue and PAS procedures were combined, the internal limiting membrane of the retina and the intimately associated vitreous body were PAS-positive, but they were not stained by alcian blue. The thin film of mucoid material situated on the inner surface of the ciliary body, particularly at the ora serrata, was stained with alcian blue but not significantly by PAS. The cytoplasmic particles in the outer epithelial tissues were stained intensely blue if the alcian blue stain was applied before the PAS reaction, but there was a faint diffuse PAS-positivity of the cytoplasm in which these blue-staining granules were visualized. In only an occasional cell did the granules appear to have been unstained by alcian blue but stained by PAS.

When the PAS procedure was applied before the alcian blue, the results were somewhat different. The cytoplasmic particles assumed a more diffuse purple color and were less distinctly defined within the cytoplasm. This was especially true posteriorly where cytoplasm tended to be diffusely reddish-purple.

The staining characteristics of these epithelial cells were not altered by previous treatment with hyaluronidase or diastase.

Toward the end of the first week, the al-

cian blue-staining intracytoplasmic structures tended to be concentrated along the inner wall of the retinal epithelial cells (away from the basal nucleus) (color plate G), but the PAS reaction still revealed a rather generalized distribution of the intensely PAS-positive material. At this stage of development, the sensory cells of the retina were still virtually undifferentiated. Only extremely delicate protoplasmic strands passed between the sensory retina and the pigment epithelium. These strands were faintly PAS-positive in contrast to the intensely PAS-positive material within the epithelium, but they were more strongly alcian blue-positive and sometimes appeared to be continous with the reticular alcian blue-positive material within the pigment epithelium. Still there was no alcian blue-positive material within the sensory cells.

By the 10th day myriad minute filamentous projections were present along the inner surface of the retinal epithelium. These projections were stained strongly with alcian blue and with PAS. At this time the strongly PAS-positive intracytoplasmic particles tended to be concentrated along the inner wall of the epithelium, with fewer in other parts of the cytoplasm. The developing rods at this stage were weakly PAS-positive and they were coated with alcian blue-positive material. When the combined alcian blue-PAS stains were employed, the filamentous projections from the inner surface of the epithelium were stained blue, while the intracytoplasmic particles concentrated along the inner wall of the epithelium were stained purple.

By the end of the second week the outer segments of the rods were fairly well developed and were moderately PAS-positive. Coating all of the percipient cells from the external limiting membrane outward was an amorphous interstitial substance which stained with alcian blue but not with PAS. Thus, with the combined alcian blue-PAS method, the rods seemed to consist of a barely visible inner segment and a purple-red outer segment, both coated by pale blue interstitial substance. Along the inner surface of the pigment epithelium, particularly in places where the tissue was sectioned somewhat obliquely or tangentially, the combined stain produced a striking picture. The relatively thick, blunt, purple-red, distal ends of the rods were seen in close apposition with the inner surface of the pigment epithelium. In spite of this intimate association, the fibrillar processes which projected from the inner surface of the epithelial cells about the tips of the rods could be differentiated from the latter with ease, for they were much finer and they stained more intensely with a deeper purple color.

During the third week the PAS-positivity of the outer segments of the rods increased while that of the epithelial cells diminished. The alcian blue-staining of the inner border of the retinal epithelium also decreased even though the mucoid ground substance about the rods persisted. PAS- and alcian blue-positive particles were still present in the cytoplasm of the outer layer of ciliary epithelium and in both layers of iris epithelium.

As the rods matured the relative amount of interstitial mucoid seemed to decrease, at least in comparison with the human retina. After 21 days it was sometimes necessary to examine the mouse retina under high power to visualize the faintly alcian blue-stained ground substance about the visual cells, while in the human retina it was strikingly demonstrated with very low magnification.

Retinal acid mucopolysaccharide in rodless albino mice

Examination of the eyes of rodless albino mice revealed a distribution of acid mucopolysaccharides that was similar to that found in normal controls. During the first week and a half the staining characteristics of the amelanotic pigment epithelium and of the fibrillary processes which project toward the sensory retina from the inner surface of the epithelial cells could not be differentiated from those of the normal albino controls just described.

Even at the end of the second week, when

the degeneration of the rods and their nuclei was well under way, the pigment epithelium and its surface layer of mucopolysaccharide appeared unaltered. By the middle of the third week the rods had disappeared and the outer nuclear layer consisted of but a single layer of degenerating cells.

The cells of the pigment epithelium, however, remained intact; even their PAS- and alcian blue-positive fibrillary processes persisted. These bridged the gap created by the disappearance of the rods, and their distal ends seemingly were inserted into the outer limiting membrane of the retina.

The alcian blue-staining ground substance seemed to be much reduced and unevenly distributed. No alteration in the amount or distribution of intracytoplasmic particles giving positive staining reactions for acid mucopolysaccharides was observed in the outer layers of ciliary and iris epithelium of the rodless albino mice at any stage of maturation.

Effect of bleaching on demonstration of acid mucopolysaccharide

A comparison of the distribution of stainable acid mucopolysaccharide in the outer layer of neuroepithelium of albino mice with that of the pigment granules in the epithelium of the pigmented strains revealed a strikingly parallelism. Even in those portions of the ciliary body where most of the acid mucopolysaccharide seemed to be concentrated along the inner half of the outer layers of epithelium, so in the pigmented animals the pigment granules had a similar distribution. It was, therefore, suspected that if the pigment were removed, alcian blue might then demonstrate acid mucopolysaccharide in the pigmented animals also. Sections bleached with permanganate and stained for acid mucopolysaccharide gave a diffuse blue-staining reaction with alcian blue that was not observed otherwise; that is, there was a general nonspecific uptake of alcian blue. Nevertheless, the pigment epithelium was stained more intensely than were the other tissues (color plate I). The areas which stained most intensely were precisely those which had contained the pigment granules before being bleached (color plate J).

Discussion

This study has demonstrated that during the early postnatal stages of retinal differentiation in the albino mouse those epithelial tissues which develop from the outer layer of the optic cup are vividly stained by alcian blue, by the Rinehart-Abul-Haj method for the demonstration of acid mucopolysaccharides, and also by the periodic acid-Schiff reaction. The staining is not affected by pretreatment with diastase or by bovine testicular or streptococcal hyaluronidases. A few observations made on albino rabbit eyes revealed a similarly selective staining, but Smelser and Ozanics,[15] in their excellent study of the distribution of radioactive sulfate in the New Zealand white rabbit, did not observe any concentration of S_{35} in the pigment epithelium or about the sensory cells. For this reason Smelser[16] suggested that if the material revealed so vividly by alcian blue and colloidal iron were, in fact, an acid mucopolysaccharide, it probably is a nonsulfated type. Thus, our own studies, coupled with those of Smelser, would indicate that the mucoid material in which the percipient elements are embedded is neither hyaluronic acid nor one of the sulfated mucopolysaccharides.

Of interest, but questionable relevance, is the report by Clayton and Feldman[17] describing the selective localization of "antigens" reacting with anti-lens antiserum in the pigment epithelium and outer part of the rods of week-old albino mice. Their observation is cited here only to call attention to the similar distribution of the "unknown" with which they were dealing and that of the "unknown" with which we are concerned.

The significance of our observations is not known, but two suggestions may be offered:

1. It is possible that the acid mucopolysaccharide is a kind of cement substance designed to keep the inner and outer layers of the optic cup in close apposition. The fact that the staining intensity is greater in the

ciliary body and iris where the layers are in much firmer union than posteriorly would seem to be consistent with this suggestion.

2. It may be that this acid mucopolysaccharide is in some way related to the biochemistry of retinal melanin, since the distribution of the intracytoplasmic mucopolysaccharide in the albino is so similar to that of the pigment granules in pigmented mice. That positive staining reactions are obtained with alcian blue after the pigment is bleached with permanganate would seem to be consistent with this suggestion.

Since the stainable retinal acid mucopolysaccharide in the newborn mouse is entirely within the cytoplasm of the outer epithelial layer, and since the visual cells at no time during their postnatal development appear to contain intracytoplasmic acid mucopolysaccharide, it seems only logical to assume that the mucoid interstitial substance between the sensory retina and the pigment epithelium is a secretary product of the latter. The gradual accumulation of intracytoplasmic reticular particles along the inner surface of the cells, the nuclei remaining at the base of the cells next to the choriocapillaris, is in keeping with this suggestion.

More direct evidence is obtained at about the ninth day after birth when delicate, tapering, fingerlike processes, which stain positively for acid mucopolysaccharide, appear on the inner surface of the cells. These seem to interdigitate with the developing outer segments of the visual cells. At this stage of development, however, the sensory retina, as seen in our histologic sections, has invariably come away from the pigment epithelium.

With further maturation of the visual cells, their outer segments become so intimately interlocked with the fine reticular cytoplasmic processes from the pigment epithelium, which, possibly because of their sticky nature, tend to keep the retina in its anatomic position even after sectioning. In fact, we have frequently observed in many of our sections of mouse eyes during the third week of postnatal development that the outer segments of the rods tend to fracture through their midzone rather than to separate from the cytoplasmic processes, perhaps because the attachment is so firm.

This phenomenon is rarely observed in human eyes, for the sensory retina separates so readily from the pigment epithelium. The stainable acid mucopolysaccharide between the sensory retina and the pigment epithelium in the human eye seems to be much more abundant than in the mouse; at least it stains more intensely. Possibly it is because of this relative abundance of interstitial mucoid that detachments of the human retina occur so commonly as artefacts. Possibly the mucoid interstitial substance of the human retina is less sticky than that of the mouse.

The observations made in this study provided no evidence to support an idea which we once entertained as to the mechanism of retinal degeneration in the C3H and Swiss albino mice. Assuming that the interstitial mucoid substance plays a role in the exchange of nutrients and catabolites between the choriocapillaris and the visual cells, we considered the possibility that the hereditary retinal degeneration in these mice might actually represent the effect of a primary disturbance in the epithelial cells. We could find no evidence to support this hypothesis, for the observable alterations in the interstitial mucoid substance appeared to follow, rather than precede, the retinal degeneration. These observations, then, would support the views of Tansley and Noell, since they are in keeping with the belief that the retinal degeneration is the result of a fairly direct effect of a mutant gene upon the growth of the sensory organelle.

Although our own studies and those of Sidman would seem to establish that the cells of the pigment epithelium are the secretors of the interstitial mucoid about the sensory cells in the mouse, this need not necessarily be the case in other species. In fact our observations that the neoplastic rosettes of retinoblastomas and the non-neoplastic rosettes of dysplastic retinas contain an apparently

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similar mucoid material would seem to be proof that the visual cells of the human retina are capable of secreting such a substance. In these lesions of the human retina, there is no participation by cells of the pigment epithelium. The very character of the lesion as illustrated in Color Plate D indicates the mucoid must be derived from the retinoblastoma cells.

Summary and Conclusions

1. The postnatal development of the tissues derived from the inner and outer layers of the optic cup in pigmented and albino mice has been studied histologically with the aid of special stains for mucopolysaccharides.

2. The aim of this study has been to obtain more information about the recently demonstrated mucoid interstitial substance which coats the visual cells.

3. Observations made on the albino mouse, whose "pigment epithelium" is amelanotic, suggest that the interstitial mucoid is derived from (secreted by) the epithelium onto the surface of the developing visual cells. This process begins early, at about the end of the first week when the differentiation of the visual cells has just begun. Later, when the outer segments of the rods are well differentiated, fine alcian blue-positive cytoplasmic particles appear to project from the "pigment epithelium" toward the rods, the tips of each being intimately interdigitated.

4. The distribution throughout the outer neuroepithelial layer of intracytoplasmic particles, which stain vividly with alcian blue, colloidal iron, and PAS in the albino mouse, is remarkably similar to that of the pigment granules in pigmented mice. After bleaching, alcian blue staining of the outer neuroepithelial layer of the pigmented mouse eye is comparable to that obtained in the albino mouse eye.

5. These morphologic studies suggest that the intracytoplasmic particles in the tissues derived from the outer layer of the optic cup may be concerned with either or both of the following: (a) the secretion of interstitial mucopolysaccharide about the visual cells; (b) the biochemistry of retinal melanin.

6. Observations made on the developing eyes of rodless mice would seem to confirm the opinions of other investigators that degeneration of the visual cells before they have completed their postnatal differentiation is the direct expression of a mutant gene and that alterations in the pigment epithelium and interstitial mucoid develop secondarily.

7. The observations made and conclusions drawn in this study do not exclude the possibility that visual cells of the human retina produce the interstitial mucoid material between the sensory retina and the pigment epithelium.

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Discussion

George K. Smelser (New York): This paper is an answer to those who have assumed that morphologic explorations are no longer rewarding. Dr. Zimmerman has, by application of modern histologic methods, brought to our attention heretofore unknown anatomic features of the eye. He suggests that the alcian blue-stained material found between the pigment epithelium and the external segments of the rods is a mucopolysaccharide, and that its function may be to cement the rod processes and the filaments of the pigment epithelium.

There can be no doubt of the importance of a material filling the space through which the exchange of substances necessary to the visual cycle takes place. Very little is known of the materials which occupy this area. The late Professor Detwiler demonstrated that droplets (Kolmer's droplets) found there were closely related to the visual function of the rods, and were derived from the rod outer segments. It is difficult to determine on the basis of staining reactions the precise nature of the stained compound. In this case, we may safely conclude that the substance is strongly acidic and we know that mucopolysaccharides are such strongly acidic compounds. As the authors have shown, this material resists digestion with hyaluronidase so that it may be concluded that it is neither hyaluronic acid or chondroitin sulfate A.

Radioautographs of our own[1] and of Dr. Godfred Larsen[2] (presented at this meeting) clearly show that the alcian-blue positive regions are relatively free from sulfate, indicating that the substance in question is not a sulfated mucopolysaccharide. Some studies have been made of this region with the aid of the electron microscope. These tell us that the alcian blue-stained zones contain a material which is electron dense. Very little material is found around the rod processes but that appears homogeneous, which is to be expected on the basis of Dr. Zimmerman's report. The cytoplasm of the pigment epithelium has been demonstrated by Porter[3] to be also dense to electrons, but no organelles have been shown which suggest themselves as the site of the alcian blue-stained material.

The function of the acidic substance may serve, as Zimmerman suggested, to cement the retinal layers together. Detachments of the retina remind us of the importance of such a glue. The two epithelial layers of the retina in the ciliary region, which rarely separate, are joined together much more effectively than elsewhere by mechanical means (desmosomes and interdigitations) as shown in preparations presented at this meeting by Dr. Pappas and myself.

In the present paper a conscientious effort was made to determine whether the alcian blue-stained substance was derived from the rod process or from the pigment epithelium. A report by Dr. Myra Johnson[4] may provide another means of critically examining this problem. Dr. Johnson separated the portion of the optic cup, from which the future pigment epithelium would be derived, from that which produces rods and cones. The retina developed normally when grafted into a new location in the developing embryo; the rods were found to be histologically normal, and Kolmer's droplets were present. It would be very exciting to learn if, when they developed free from the pigment epithelium, thy are also free from the strongly acidic material reported by Dr. Zimmerman.

Dr. Lorenz E. Zimmerman (closing): In closing, I just want to re-emphasize the point that I made (but perhaps not strongly enough in the paper) that actually we don't know what we are staining, and we speak of acid mucopolysaccharides simply because this material stains the way acid mucopolysaccharides should stain. We don't really know what the material is, nor do we know whether it is one thing or a number of things—a mixture.

There are some reasons for believing that perhaps in different locations there is a mixture, that the material actually present in the interstitial space around the rods may be produced by the pigment epithelium, but that the material which we see remaining within the cells of the outer neural epithelial layer may be something still different and may be totally unrelated, as the bleached preparations suggest; that is, it may be something that is related to pigment metabolism in that particular instance.

I want to thank Dr. Smelser for kindly discussing the paper and bringing the added information to us.

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INDUSTRIAL TEAR STUDY*

Filter-paper electrophoresis of tears, with results of an industrial study from 1,000 specimens sent by mail

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In the spring of 1957, the California Department of Public Health, Bureau of Adult Health, Berkeley, California, asked our assistance in a study concerned with occupational exposures to low concentrations of certain gases commonly produced in the combustion of hydrocarbon fuels. One of the complaints of the men was tearing and irritated red eyes. We were a part of the consulting medical team to study the amount and composition of tearing since this could be correlated with the research project which we were undertaking.

Tear collection analysis

Previously we had devised tear kits which included: (1) a millimeter measuring scale; (2) Schirmer papers made of five mm. width pH hydrion paper folded down five mm. in paper packets which had been left in the oven one-half hour at 300°F.; (3) Saran triangles, so that the tear specimens could be immediately wrapped to preserve their moisture while they were being sent by special delivery mail to our laboratory; (4) plastic film for double seal with Scotch tape.

Most of the runs were completed within 48 hours after the specimen was taken. Because the volume of these specimens was far beyond our capacity to complete immediately and because they happened to come when Beckman was revising the Analytrol, our immediate reports consisted of calculations of the rate in lambda per minute and an impression report of the concentration of the three proteins, lysozyme, globulins, and albumins. We graded them absent, deficient, low, medium, and high. By the time we had completed all the runs, kept our regular work current, and the Analytrol had been revised, three months had elapsed. In estimating the number of grams present we allowed one-third for fading. The fading of the three protein components appeared to be uniform. The figures which we present, therefore, are not exact but proportional and point up a possible method for future use and the general area in which these figures fall. (In a serum control using this method we found an average 6.7 BEL [Bromphenol blue equivalents of lysozyme] gm. per 100 cc. which is a reasonable concentration of a normal serum.)

Grading of rate

In view of the fact that known medical criteria were expected in this study we felt that rate was the most important. (Schirmer tests have been used since 1903.) We had originally classified all rates below 2.5 deficient (Schirmer's level of normal) and from

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TABLE 1

SIGNIFICANCE OF RATE CHANGE

Job No.	Decreased	Increased	Same	Significance Level
1	18	7	24	0.02*
2	17	4	11	0.00+†
3	11	4	7	0.06
4	10	3	9	0.05*
5	34	5	11	0.00+†
6	10	4	8	0.09
7	12	3	6	0.02*
	112	30	76	0.00+†
Time No.				
1	4	1	4	0.19
2	9	2	5	0.03*
3	11	4	5	0.06
4	36	7	17	0.00+†
5	25	8	19	0.00+†
6	27	8	26	0.00+†
	112	30	76	0.00+†

GRADING OF TEAR SECRETIONS

Categories Significant at	Jobs	Times
5% level	1, 2, 4, 5 & 7	2, 4, 5, 6 & totals
1% level	2 & 5	4, 5, 6 & totals

* Significant.
† Highly significant.

2.5 to 3.0 low, 3.0 to 10 medium and above 10 high. However, we found a majority of the workers fell into the deficient group so we subdivided this group into: 2.0-2.5, high deficient; 1.0-2.0 low deficient; and below 1.0, deficient. Table 1 shows the number who increased, decreased, or remained in the same grade by job number and time.

Following the extensive experience with the Schirmer test in co-operation with the California Department of Public Health, we would like to suggest Table 2 as a new type of grading of tear secretions. Schirmer and other workers have reported less secretions in men. Probably the majority of cases as low as 12-mm. Schirmer (2.0 lambda/minute) have a normal protein distribution. This table, therefore, was devised from the present experience for our future research. Because 17-mm. strips absorb 10 lambda (0.01 ml.), which is convenient in metric calculations and gives an adequate amount of proteins to fit in the framework of our micro method of analyses, they were used instead of 35-mm. Schirmer. The 17-mm. strip centers the protein on a standard 30-mm. electrophoresis strip so that the entire dyed protein passes through the photocell for calculating total proteins. The conversion table (table 3) was designed to save time in making reports and to promote consistency in our punch-card system.

GRADING OF PROTEINS (fig. 1)

For the purpose of this study we recorded the amount of proteins produced by a 17-mm. Schirmer. If this was moistened in less than five minutes it was removed from the eye and the specimen taken for protein analysis by electrophoresis as described. If it was not possible to obtain 10 lambda in five minutes, the volume was mathematically corrected to 10 lambda for protein calculation.

The total specimen was graded for the purpose of this study by the height of the scan using a 600 millimicron interference filter in the Analytrol, bromphenol blue dye on S & S paper No. 2043A mgl. paper strips. As shown by Figure 1, less than 1.0 cm. was graded deficient; 1.0 to 3.0, low; 3.0 to 6.0, medium; 6.0 to 10, high; above 10, high plus.

Figure 2 shows the percentage of the 888 analyses as graded by the standards of Figure 1. In 36 percent of the cases lysozyme was below normal (nine percent of these showed no lysozyme by this method). In 48 percent the height of the curve was between 1.0 and 3.0 cm. and was classified as low, while 14 percent between 3.0 and 6.0 cm. are classified as medium, and only two percent are high. Ten percent of the globulin curves are below 1.0 cm. and 12 percent of the albumins. This graph shows that lysozyme is the first of the proteins to decrease. A half-percent lysozyme control reached 4.5 cm. (5.0 µg./lambda).

Figure 3 shows the difference in tear proteins between the beginning and end of the day for one of the workers.

TABLE 2

CONVERSION TABLE FOR THE 17-MM. SCHIRMER PAPERS

(Based on 0.6 lambda per mm.)

	Grade		MM. of Schirmer	Minutes	Seconds	Rate Lambda/min.
Psychic tearing	10		17	—	—	—
Above normal	9		17	0	15	40
Normal	8	High	17	0	30	20
			17	0	45	15
	7	Medium	17	1	0	10
			17	1	7	9
			17	1	15	8
			17	1	26	7
			17	1	40	6
			17	2	0	5
	6	Low	17	2	30	4
			17	2	51	3.5
			17	3	2	3.3
			17	3	20	3.0
Limit range	5		17	3	34	2.8
			17	3	51	2.6
			17	4	0	2.5
	4		17	4	10	2.4
			17	4	21	2.3
			17	4	33	2.2
			17	4	46	2.1
			17	5	0	2.0
Subnormal	3	Minimal	16	5	0	1.9
			15	5	0	1.8
			14	5	0	1.6
			13	5	0	1.5
	2	Moderate	12	5	0	1.4
			11	5	0	1.3
			10	5	0	1.2
			9	5	0	1.0
	1	Marked	8	5	0	0.9
			7	5	0	0.8
			6	5	0	0.7
			5	5	0	0.6
			4	5	0	0.4

Figure 4 shows the classification into which the lysozymes, globulins, and albumins fell before and after work. It will be noted that in all three of the proteins more specimens fell into the subnormal group after work and that most of them are in the low group according to this classification.

Figure 5 shows that the protein concentration in spite of its many other variables is fairly constant and stable. 98 percent had a concentration of 0.4 to 2.9 gm. per 100 cc. Of these 81 percent were either one or two gm. In this industrial series there were none with extremely high concentrations.

Figure 6 reports the albumin/lysozyme ratio in an attempt to correlate the pH with the protein balance. Most people complain of burning eyes if the ratio is above 3.0 (three parts albumin one part lysozyme). Because of the difficulty in separating the two albumins we felt that a relative increase in the serumlike albumin was the first subtle change to take place with stress, and that it is possibly caused by failure of the lacrimal

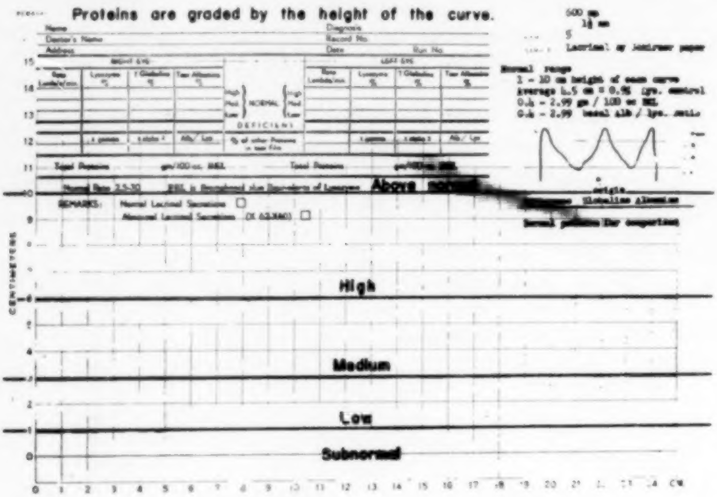

Fig. 1 (Erickson, et al.). Grading of proteins.

TABLE 3

BEL AND LAMBDA CORRECTIONS

(Based on BEL 0.0067; based on 10 Lambda)

Total Speci-men (mm.)	Multiply Integration Units in Scan	BEL-Lambda =gm./100 cc.
20		0.0056
19		0.0059
18		0.0065
17		0.0067
16		0.0067
15		0.0074
14		0.0080
13		0.0087
12		0.0094
11		0.0100
10		0.0114
9		0.0120
8		0.0104
7		0.0160
6		0.0187
5		0.0221
4		0.028
3		0.038

Example: (See Figure 3)

$$20\text{ mm. } 183 \text{ integration units} \times 0.0056 = \frac{1.0\text{ gm.}}{100\text{ cc.}}$$

$$8.0\text{ mm. } 15 \text{ integration units} \times 0.0104 = \frac{0.2\text{ gm.}}{100\text{ cc.}}$$

gland to produce an enzyme which performs this function of modifying the albumin structure.

When we were approached about this industrial study, we were asked if we had a single simple answer to tear secretion. Protein studies are never simple but if we begin with two known accepted protective mechanisms of the eye, (1) the washing powers as expressed by rate and (2) the lysozyme content expressed in μg., we would have one significant answer.

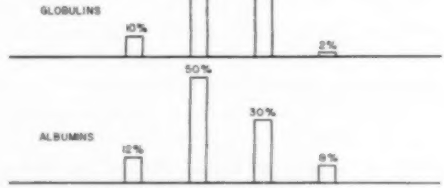

Fig. 2 (Erickson, et al.). Distribution of tear proteins in 888 industrial analyses.

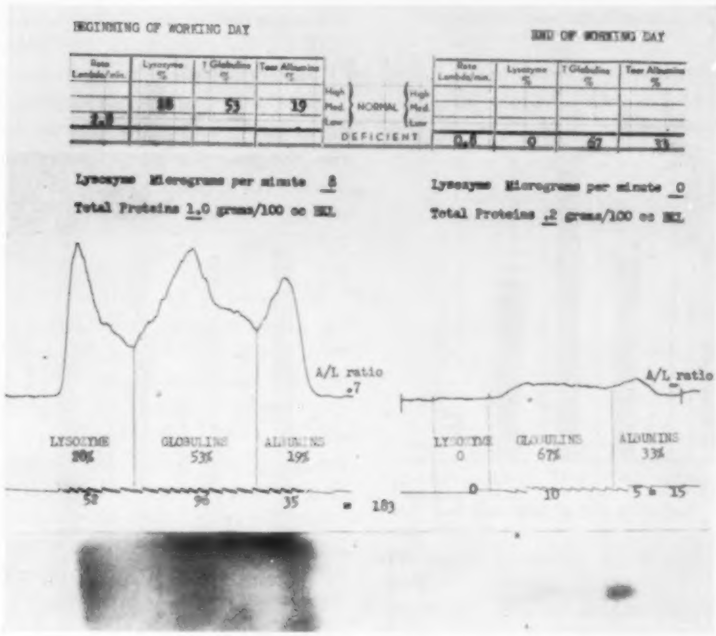

Fig. 3 (Erickson, et al.). Change in tear proteins.

Lysozyme is an enzyme so a little goes a long way. It is our clinical impression from experience based on about 4,000 analyses (with phosphate buffer we have done approximately 2,000 and with barbitol buffer [Spinco B2] we have done over 2,000) that almost all patients showing 1.0 µg./min. during any part of the day have eye complaints and objective findings such as burning, red-

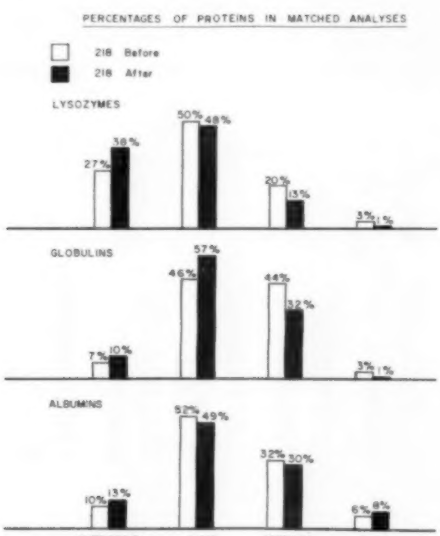

Fig. 4 (Erickson, et al.). Percentages of proteins in matched analyses.

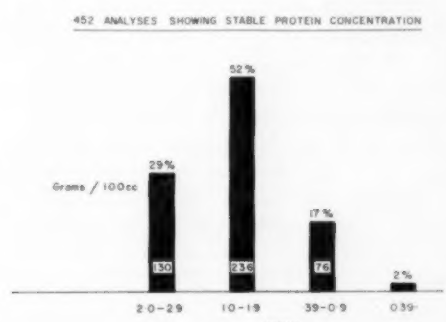

Fig. 5 (Erickson, et al.). A total of 452 analyses showing stable protein concentration.

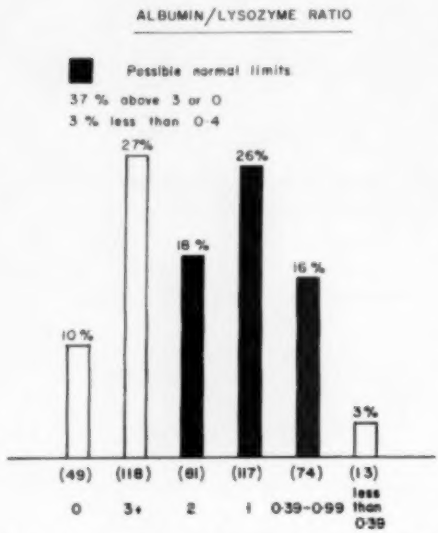

Fig. 6 (Erickson, et al.). Albumin/lysozyme ratio.

ness, or plugged tear ducts. This may be due to insufficient lysozyme. A decrease in lysozyme significantly alters the pH and may cause the burning sensation.

To verify this we did some lysozyme control studies and devised the method of calculation as shown on Figure 7 which demonstrates the method used to calculate the number of µg. of lysozyme per minute from total proteins in gm. per 100 cc. BEL (bromphenol blue equivalents of lysozyme).

The original example shows 0.7 gm. per 100 cc. which is equivalent to 7.0 gm. per 1,000 cc. By multiplying 10^{-6} the answer is 7.0 µg. per lambda. Three percent of the proteins in this case was lysozyme, therefore, $7 \times 0.03 = 0.2$ µg. per lambda. In this case also the patient's rate of secretion was 1.0 lambda per minute. Therefore, 0.2 µg. of lysozyme per lambda times one lambda per minute equals 0.2 µg. of lysozyme per minute.

In Figure 8, the horizontal line gives the age group by tens. The analyses of the men were divided into three by their ability to produce lysozyme in µg./min., (1) below 2.0, (2) 2.0 to 10, and (3) 10 to 125. There was very little difference in any of the age groups in the below 2.0 category. All of them average 38 percent. About 20 percent below the age of 40 years produce 10 to 125 µg. of lysozyme per minute while above the age of 40 years only 15 percent produce these more adequate amounts.

In Figure 9, the graph shows the difference by age groups before and after work. It will be noted that in the 20-year-old age group none was extremely deficient in the secretions at the beginning of the work period, while 22 percent fell into this group after work.

Figure 10 shows the secretion of lysozyme

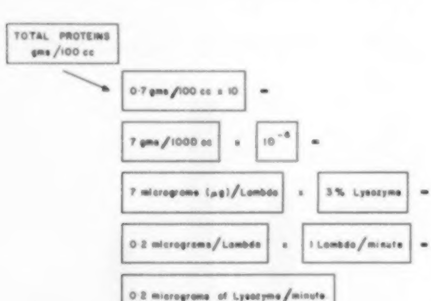

Fig. 7 (Erickson, et al.). Calculations for lysozme in gm./min.

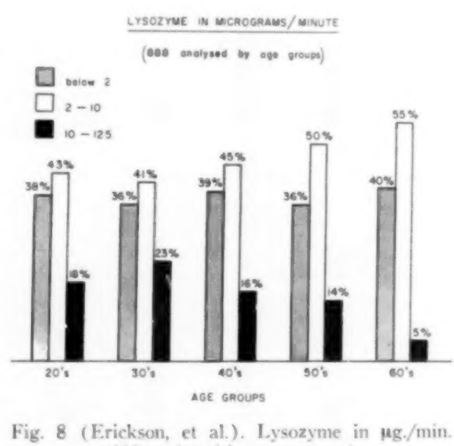

Fig. 8 (Erickson, et al.). Lysozyme in µg./min. (888 analyzed by age groups).

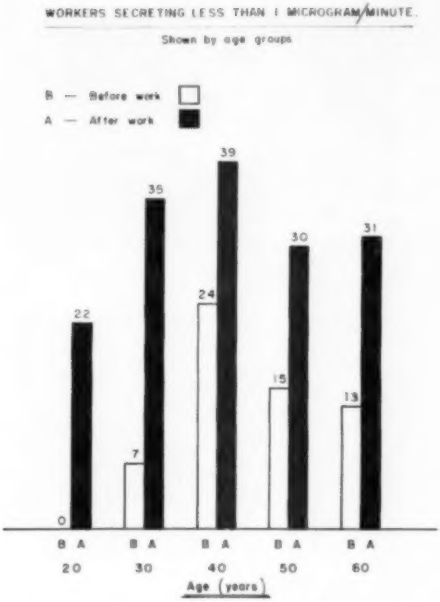

Fig. 9 (Erickson, et al.). Workers secreting less than 1.0 µg./min. before and after work (shown by age groups).

in µg./min. It will be noted from Figure 10 which compares these data to 50 normals that 24 percent of this total had less than 1.0 µg./min. compared to four percent in the control

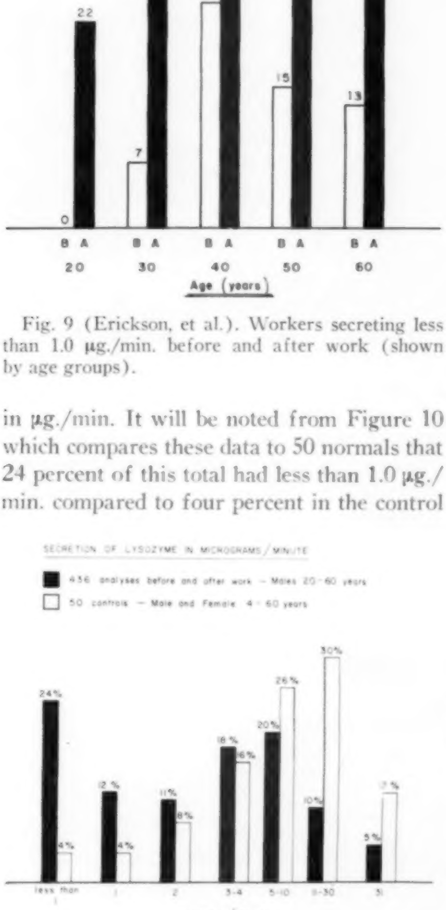

Fig. 10 (Erickson, et al.). Secretion of lysozyme in µg./min. Comparing 436 analyses with 50 controls.

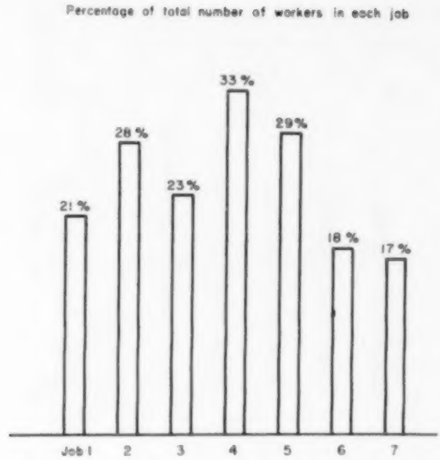

Fig. 11 (Erickson, et al.). Lysozme in µg./min. below 1.0 by job.

group; in the 11 to 30 µg./min. group 30 percent of the control group as compared to 10 percent of the men of the industrial study.

These figures and graphs point out very clearly that a much greater percentage in the group of industrial workers was producing an insufficient amount of lysozyme. The normals consisted of a group of men and women, mostly city workers and housewives, who came in for routine refractions and had no eye symptoms.

Figure 11 shows lysozyme in µg./min. below 1.0. These data are taken from Table 2 and graphically illustrate the lowering of resistance at the end of the working day and show the effect of fatigue. For countless ages it has been known that tearing tends to wash away foreign substances from the eye. More than 50 years ago Dr. Fleming described the protective functions of lysozyme and since that time his observation has become an accepted fact.

Figure 12 shows the striking increase in the markedly subnormal production of less than 1.0 of lysozyme in µg./min. In Job 5, for instance, at the beginning of a working period, 18 percent were in this group while at the end of the working period 40 percent.

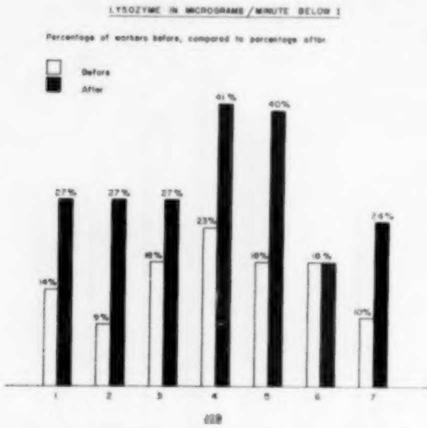

Fig. 12 (Erickson, et al.). Comparison before and after work by jobs of men who produced less than 1.0 µg/min.

The workers on this job had most exposure to Diesel fumes, but even the men in Job 1 who were not exposed comprise a significant increase in this low group. Also in the matched group was the total number in each job which fell below 1.0 regardless of time. In Jobs 2, 4, and 5, for example, 28, 33, and 29 percent, respectively, fell into this extremely low lysozyme-producing group.

Figure 13 is a normal protein separation using phosphate buffer and heavy electrophoresis paper, the technique used prior to June, 1957. The same technique was used for Figures 13, 14, 16, and 17.

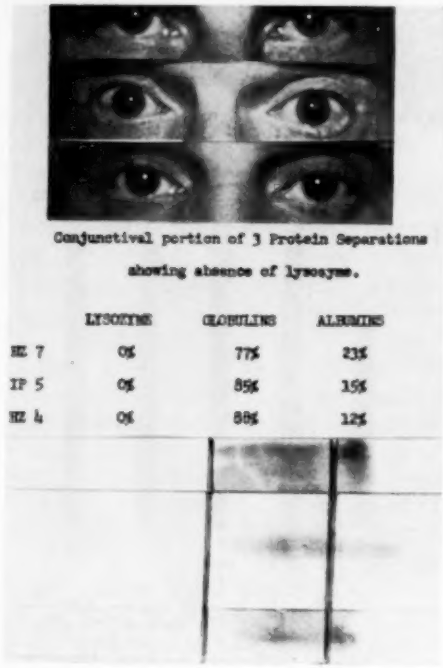

Fig. 14 (Erickson, et al.). Dilated pupils associated with absence of lysozyme.

Before the major study was undertaken about 150 trial runs were made. At that time Kodachromes of the eyes were taken (fig. 14). The lighting conditions were the same for all the pictures. The purpose of this study was to evaluate the conjunctiva for in-

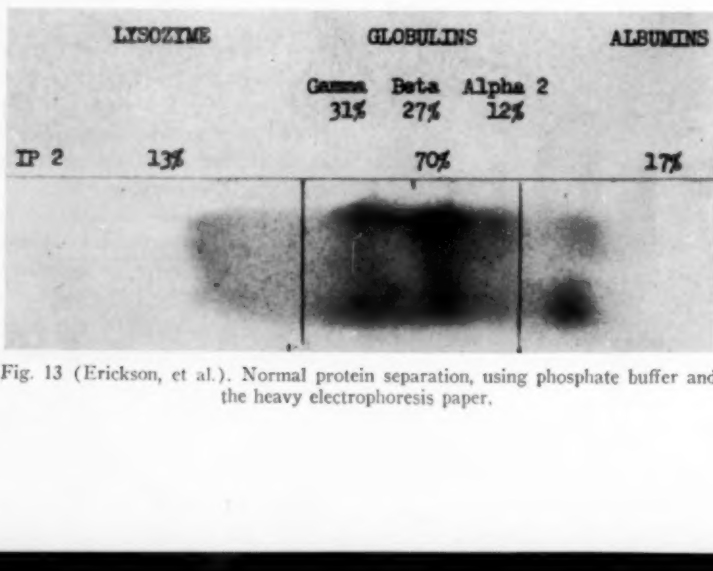

Fig. 13 (Erickson, et al.). Normal protein separation, using phosphate buffer and the heavy electrophoresis paper.

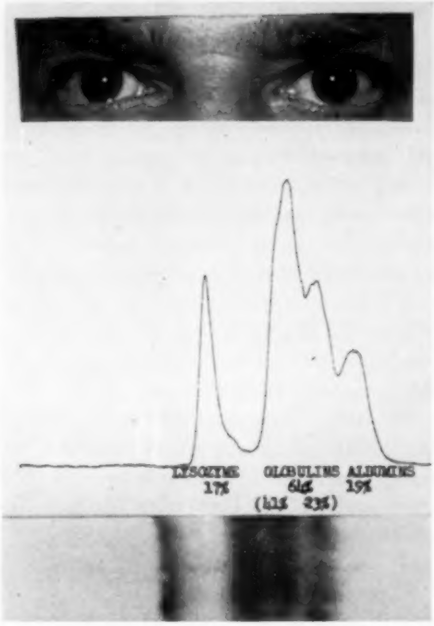

Fig. 15 (Erickson, et al.). Dilated eyes associated with a component of alpha 2 mobility. (Referred to as component III by Dr. McEwin.)

flammation. One of the striking findings was the large number of dilated pupils. All of the Kodachromes were placed in a viewing box and measured. Four pairs of pupils measured wider than any others. Of these, three showed no lysozyme at all. This may prove to be of significance, particularly in future glaucoma studies, since these observations suggest that fatigue produces a scopolamine-like reaction. To compare, we picked out three of the tiniest pupils and found that, even if the tear rate was low, the protein balance was more normal, with an adequate amount of lysozyme. The percentages found in each group are shown in Figure 17.

Figure 15, which shows the fourth wide-pupil strip, is very interesting because it demonstrates an extremely high alpha 2.

The pharmacology classes co-operated by taking tear specimens before and after autonomic drugs by mouth. In Figure 16, typical responses are shown for atropine and scopol-

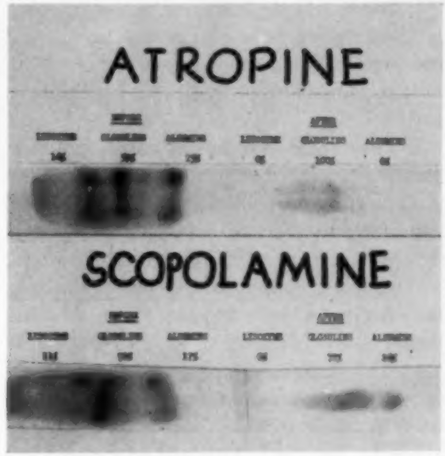

Fig. 16 (Erickson, et al.). Response in tear protein production following atropine and scopolamine by mouth.

amine. Note the great similarity in the response to fatigue and to scopolamine.

Summary

1. The collection of tear proteins can be done simply and effectively by preserving their moisture in Saran Wrap and sending them by mail. We have analyzed satisfactory samples sent from the Panjab State of India and from the Belgian Congo. In this industrial study, 1,200 specimens were sent to our laboratory by special delivery mail.

Fig. 17 (Erickson, et al.). Miotic pupils associated with presence of lysozyme.

2. The technique described, employing the standard electrophoresis apparatus which has been developed for the separation of blood proteins, can be used by a standard laboratory.

3. This technique introduces a possible method not only of evaluating the percentage of each protein, but also of determining quantitatively their concentration. Because each protein has its own particular dye affinities, we have choosen to use lysozyme as the basis for comparison and express it as bromphenol blue equivalents of lysozyme (BEL).

4. By calculating from rate, from percentage of lysozyme, and from concentration, we are now able to give one simple answer which we feel may be significant not only as regards this protective mechanism of the eye, but also as an expression of the individual's ability to produce other secretions.

Conclusion

In conclusion, I would like to extend my thanks to Dr. William Clark, to the other health officers, and especially to Dr. Christine Einert for furnishing the Schirmer strips and the correlating physiologic data. I am most grateful to be living in a country and state, where as individuals, or as groups, anyone can come to the health department to get an honest answer to an honest question.

It didn't matter when the men came on or off duty—2:00, 4:00, or 6:00 A.M. or P.M., our health officers were there to check the blood pressure, chest, and eyes.

We also extend our thanks to the Federal Department of Health which made this investigation financially possible by way of a grant to Stanford University Medical Research.

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Clark, W. H.: Report to the Advisory Committee. California Department of Public Health, Bureau of Adult Health, Feb. 1, 1958.

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Discussion

Dr. P. C. Kronfeld (Chicago): This certainly is an elegant method for the estimation of the relative and absolute concentrations of the three principal protein fractions in small samples of tears. Having grappled with problems of component separation in protein mixtures, I won't say how many years ago, my feelings toward Dr. Erickson's method are those of admiration with a strong admixture of envy. It is, of course, fortunate that lysozyme, the lacrimal protein in which Dr. Erickson is most interested, lends itself beautifully to electrophoretic separation because of its very high isoelectric point. It is reasonable to consider lysozyme as the most specific, most representative lacrimal protein, not because of its bacteriostatic property, but because of its chemical specificity.

I found the evaluation of Dr. Erickson's results a little difficult because of the subordinate part that controls play in her paper. But she undoubtedly has made very extensive studies on control cases. In today's paper she refers to one control series of 50 office patients without complaints referable to conjunctiva or the lacrimal system. A still better control series are the pharmacology students at Stanford referred to in today's paper. The value of the latter series could be greatly enhanced if Dr. Erickson gave, in addition to the arithmetic means of the lysozyme, albumin, and globulion concentrations, some indication of the variations within this series, such as its standard deviation.

But even these scanty references to controls establish enough of a baseline to recognize the phenomenon of lowering of the Schirmer rate, lowering of the total protein output, and, possibly, a specific lowering of the lysozyme output in industrial workers during the course of their regular working day.

Whether this lowered output should be interpreted as a deficiency state, an expression of normal fatigue, or a physiologic diurnal variation I would not want to decide from the data presented today. It may very well be a deficiency state but, to prove that, more data pertaining to diurnal variations in lacrimal output of workers in other occupations would be needed, particularly occupations which do not entail exposure to external irritations. I would also look for correlations of lowered protein output with other chemical or cytologic properties of the

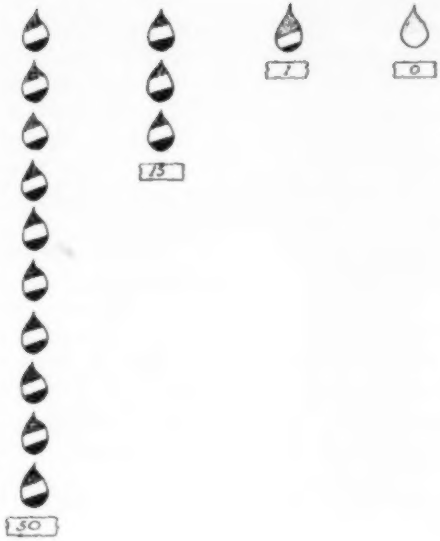

Fig. 18 (Erickson, et al.). Graphic illustration of protein content of lacrimal secretions.

The first row of 10 tear drops illustrates a normal rate of flow. The composition of each represented by 30-percent albumin (dotted), 40-percent globulins (the clear center), and 30-percent lysozyme (the black).

When the rate drops to three lambda, the proportions of the protein remain the same. One lambda, however, as represented by one drop, shows a relative decrease in lysozyme. Six tenths of a lambda is represented by the last drop which has no lysozyme. The number at the bottom is the µg./min. of lysozyme one would expect at each rate.

tears. Paper electrophoresis at a different pH might be helpful.

From my admittedly schoolmasterly point of view, Dr. Erickson's findings in control individuals before and after systemic administration of cholinergic inhibitors are most interesting and significant.

Dr. Erickson's method I believed has a big future in the study of: (1) potential external, atmospheric irritants, (2) in the study of the common clinical entity of conjunctival irritation and discomfort without signs of infectious or allergic disease, and (3) in the restudy of general lacrimal physiology. The findings obtained so far are valuable additions to our knowledge of lacrimal physiology.

Dr. Olive F. Erickson (closing): Thank you very much, Dr. Kronfeld. The students' studies were not included because they were done by a different method. We changed from a barbital buffer, and there were a lot of changes in our equipment, so we felt we could not compare these two groups.

You are absolutely right; we should have more controls, but we were so busy doing this work that we didn't have time to do others. However, we did use some workers who were not exposed, and they showed the same thing. It was definitely a fatigue pattern.

(Slide, fig. 18) This is a repetition of what is on the blackboard, to give you some idea of what the approximate µg./min. are in the normal and what has been considered subnormal—50 would be a medium normal according to our present ideas; approximately 15 would be a low normal; I think 1 would be quite subnormal but, as we have seen from this study, there are many who get along with much less.

This large number of working men show that many people get along with little lysozyme. This group complained of burning and chronic irritation and, when they were examined, they were found to have chronically red eyes.

THE MAST CELLS IN THE UVEAL TRACT OF THE EYE AND CHANGES INDUCED BY HORMONES AND AVITAMINOSIS-C*

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New York

The mesenchymal uveal tract of the eye is known to contain numerous vessels. Besides the vessels the uvea contains similar elements as does all connective tissues, that is, cells, structural components, and ground substance. The cells are fibroblasts, histiocytes, lymphocytes, and mast cells, and characteristic for the uvea are the pigmented chro-

* From the Department of Ophthalmology, College of Physicians and Surgeons, Columbia University. Presented before the Eastern Section of the Association for Research, Washington, D. C., January, 1958.

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matophores. Of the structural components the elastic fibers are especially abundant. The ground substance consists of proteins and mucopolysaccharides, the nature of which very little is known. The mast cells of the eye have previously been studied only sporadically (Jorpes, et al., 1937[1]). They found mast cells in the iris, the ciliary body, in the episcleral limbal tissue, and conjunctiva. Accumulations of mast cells have been observed in the iris and the ciliary body in glaucomatous iritis (Holmgren and Stenbeck, 1940;[2] Zollinger, 1949;[3] and Asboe-Hansen, 1950[4]).

In this study the normal distribution of the mast cells and their behavior in response to endocrine and vitamin deficiency influence were investigated.

Material and Methods

Various species of animals were used in the experiments, namely 100 guinea pigs, 30 rabbits, 38 rats, and seven mice. All of them were male and albino. The weights of guinea pigs varied from 340 to 500 gm., those of the rabbits from 2,440 to 3,050, those of the rats from 200 to 270, and finally, the mice from 20 to 27 gm. The animals were submitted to the following treatment:

Cortisone, thyroidectomy, thyroidectomy plus thyrotrophin,‡ thyroxine, and vitamin C-free diet§, respectively, and divided into groups, five to seven animals in each group. All injections were given intraperitoneally. Animals with remnants of thyroid tissue were excluded. All the animals were killed with ether about 18 hours after the last injection. To eliminate differences in technique as far as possible, four groups of guinea pigs (included in the groups above) were submitted to the treatment just mentioned (except vitamin C-free diet) and treated at the same time. These animals were killed at the same time, fixed in the same solutions, stained with the same samples of staining solutions, and so forth. Fourteen guinea pigs, five rabbits, six rats, and one mouse were used as controls. Of the guinea pigs, 23, not included in the given numbers, died during the experiments, most of them from the vitamin C deficient diet or after the thyroidectomy operation; few after treatment with thyroxine. Five rats and three rabbits died after the thyroidectomy.

Whole eyes or sections of eyes (consisting of rectangular pieces cut from the region of the optic nerve forward into the cornea) were fixed in 4.0-percent lead subacetate or 0.4-percent 5-amino acridine hydrochloride in 50-percent ethanol through 24 hours, then dehydrated, embedded in paraffin, and cut into sections of 6.0 μ; following deparaffinization the sections were stained with 0.5-percent toluidine blue for 20 minutes, hematoxylin-eosin or the PAS-method, dehydrated, covered with coverslips and Canada balsam, and examined. No attempt was made to count the exact number of mast cells because of the special arrangement of the uveal tissue in the eye and the many errors involved in such a calculation, but the quantitative estimation was based on examination of several sections, of which 50 were examined by the author without knowing which treatment was effectuated on the particular specimens.

Results

The normal uvea showed mast cells in varying amounts in the same species of animals. In the various species of animals the guinea pigs contained in average most mast cells (fig. 1), then in decreasing numbers, the rats (fig. 2), the mice, and finally the rabbits (fig. 3). The distribution of the cells was invariably so that by far the highest number was found in the choroid (figs. 1, 2, and 3), especially in the posterior and thicker part of this membrane; in the ciliary body considerably fewer mast cells were present (fig. 4). In the iris only very few sections contained

‡ Kindly supplied by Parke, Davis and Co.

§ Rolled oats, 25 percent; dried skimmed milk, 30 percent; wheat-bran, 45 percent; the ingredients are mixed with salt and water and baked to make crackers. Vitamin A and D concentrates were given twice a week.

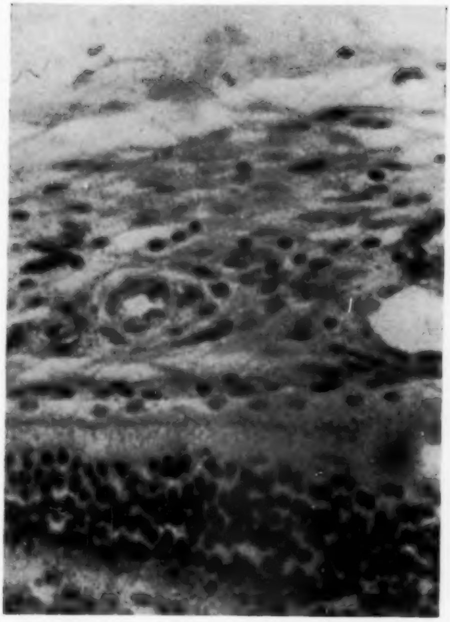


Fig. 1 (Larsen). Normal, granulated mast cells in the choroid of a guinea pig. Note location around the vessels. (Toluidine blue, 0.5 percent, ×400.)

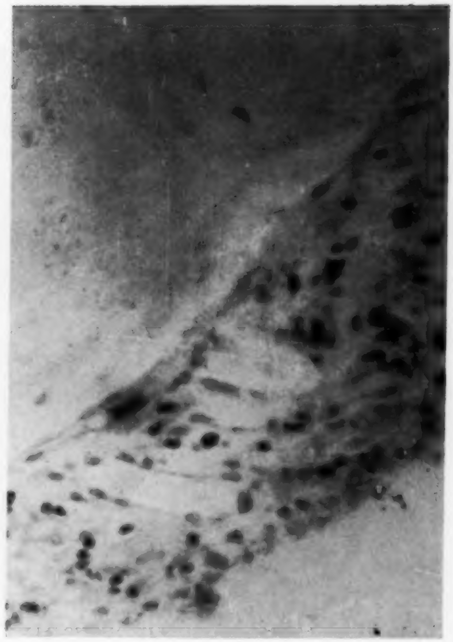
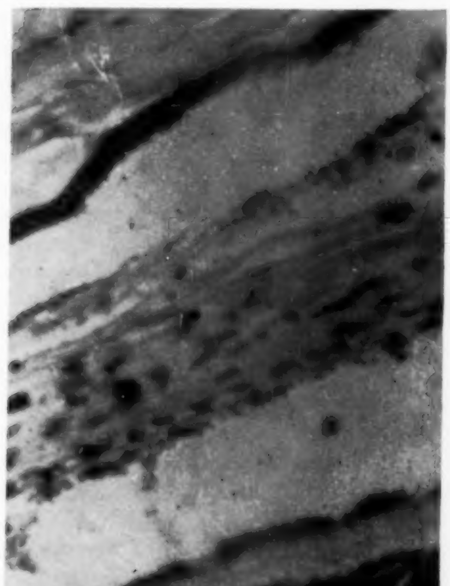


Fig. 2 (Larsen). Normal, granulated mast cells in the choroid of a rat. (Toluidine blue, 0.5-percent, ×400.)



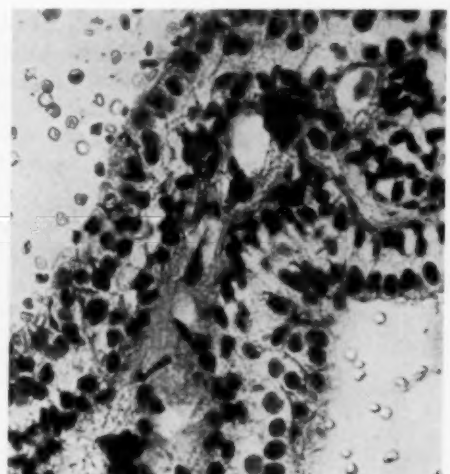


Fig. 4 (Larsen). Normal mast cells in the ciliary body of a guinea pig. (Toluidine blue, 0.5 percent, ×1,000.)

Fig. 3 (Larsen). Normal, granulated mast cells in the choroid of a rabbit. (Toluidine blue, 0.5 percent, ×400.)

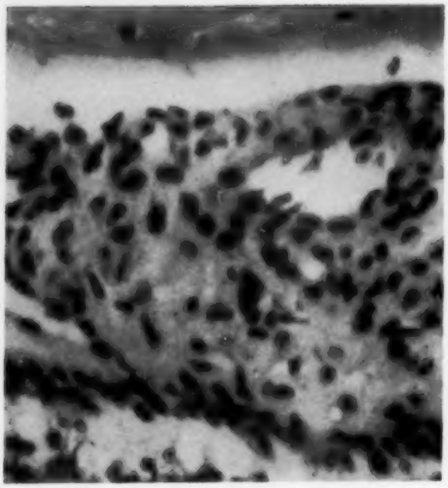


Fig. 5 (Larsen). One mast cell in the iris of a normal guinea pig. (Toluidine blue, 0.5 percent, ×1,000.)

mast cells and always near the root of the iris (fig. 5).

This distribution of the mast cells was the same in all species of animals. Nearly all of the mast cells showed considerable amounts of granules in the cytoplasm and frequently it was difficult to distinguish the single granules from each other. The sizes of the cells varied to some extent, but not considerably; the shapes of the cells seemed mostly to depend on the surrounding tissue, being more filiform in the suprachoroid layer, whereas in the other layers of the choroid more or less ovoid.

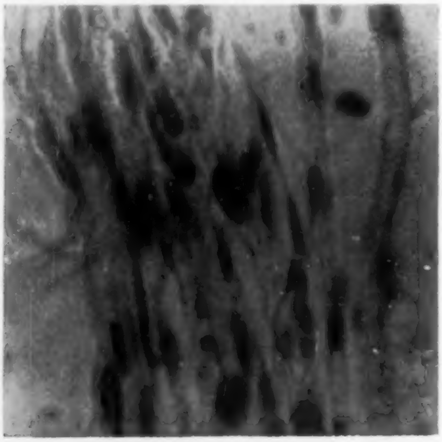


Fig. 6 (Larsen). Unaffected mast cells in the choroid of a guinea pig, treated with cortisone. (Toluidine blue, 0.5 percent, ×1,000.)

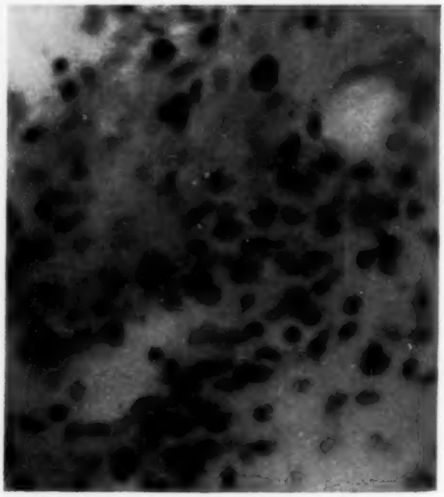


Fig. 7 (Larsen). Unaffected mast cells in the choroid of a rat, treated with cortisone. (Toluidine blue, 0.5 percent, ×1,000.)

Only small variations were encountered in the appearance of the mast cells in tissues taken from various species of animals. The mast cells in rabbits stained more orthochromatic and were less granulated (fig. 3) than those of the other species. The guinea pigs, rats, and mice did not show remarkable variations among the mast cells, neither in size nor morphology. The ground substance gave very faintly or no metachromatic stain.

In the cortisone group the guinea pigs and rabbits, which got the smaller doses of cortisone respectively (fig. 6) (10 and 25 mg.) did show none or only small changes of the mast cells. One group of rats did not show significant changes (fig. 7) except in two animals. The other group of rats showed changes in form of degranulation and vacuolization of the cytoplasm and clumping of the granules (fig. 8).

The rabbits treated with medium doses showed the changes of the mast cells men-

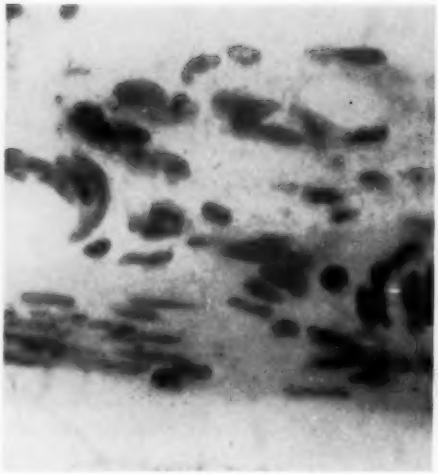


Fig. 8 (Larsen). Cortisone affected (degranulated, vacuolized) mast cells in the choroid of a rat. (Toluidine blue, 0.5 percent, ×1,000.)

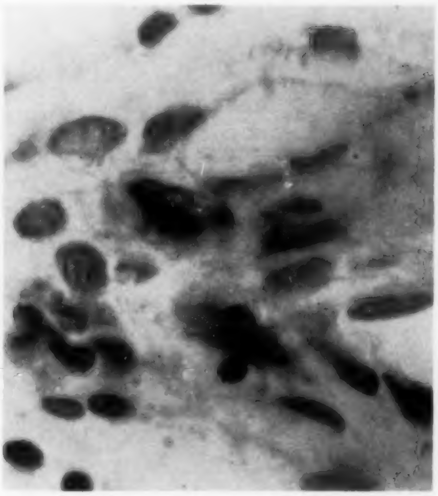


Fig. 9 (Larsen). Cortisone-affected mast cells in the choroid of a guinea pig. (Toluidine blue, 0.5 percent, ×1,000.)

tioned above, but no significant changes were noted in the guinea pigs similarly treated. Out of the groups of guinea pigs and rabbits treated with the highest doses of cortisone (15 and 50 mg.) all the rabbits gave a significant response (fig. 9); in one group of guinea pigs only some of the cells were affected in two animals, whereas the other group showed changes in almost all the mast cellls so remarkable that many of them were hardly distinguishable because of the extreme degranulation (fig. 10). This was the group which belonged to the animals treated simultaneously. Finally, in all the mice the mast cells showed significant changes. The ground substance stained only faintly with the PAS method (fig. 11).

The mast cells from the thyroidectomized animals responded in the following way. Two groups of guinea pigs showed higher numbers of mast cells, increase of size, and heavy granulation of the cytoplasm (fig. 12); one group of guinea pigs seemed not to be affected. In the rabbits only six out of 10 showed significant changes. Six out of seven rats showed similar results. The animals which were thyroidectomized and treated with thyrotrophin as well responded well to treatment (fig. 13), except two guinea pigs. The appearance of the mast cells was the same as described above, the changes being usually more significant. In this group the ground substance showed metachromasia and the PAS staining gave a pink color (fig. 14).

In the thyroxine group six out of eight rats and 10 out of 13 guinea pigs (fig. 15) showed degranulation of the mast cell cyto-

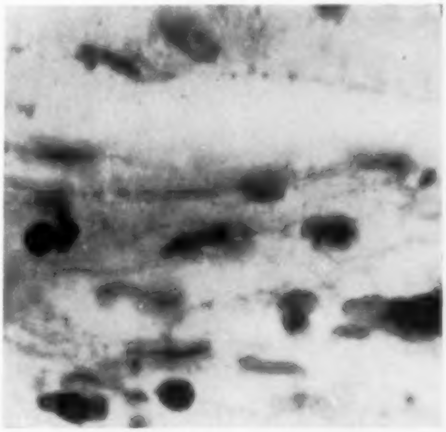


Fig. 10 (Larsen). Other cortisone-affected mast cells in the choroid of a guinea pig.

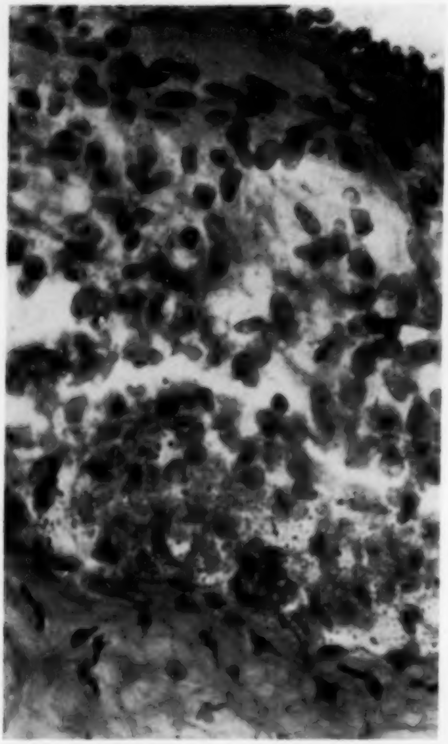


Fig. 11 (Larsen). PAS-stain of ciliary body in cortisone-treated guinea pig. No PAS-positive cells or intercellular material. (×1,000.)

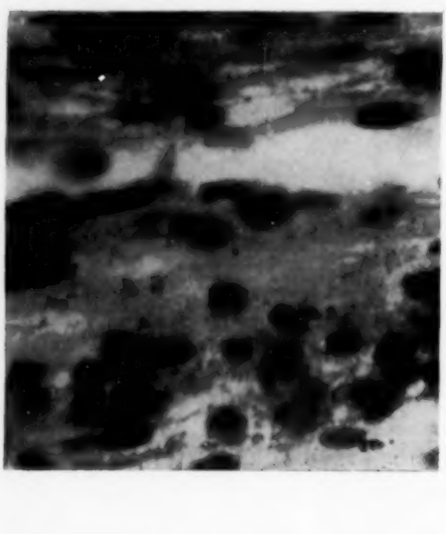


Fig. 13 (Larsen). Accumulation of mast cells in a thyroidectomized guinea pig, treated with thyrotropin. (Toluidine blue, 0.5 percent, ×200.)

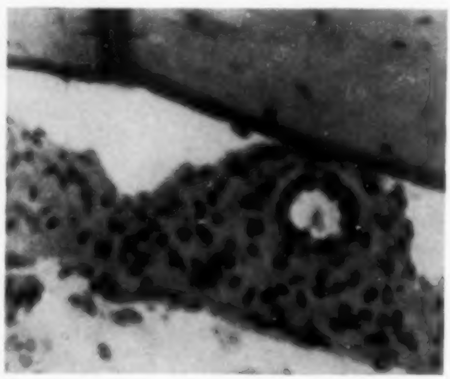


Fig. 14 (Larsen). PAS-stain of iris in thyroidectomized, thyrotrophin-treated guinea pig. PAS-positive material intercellularly. (×400.)

plasm, especially of the cells some distance from the vessels, while most of the cells close to the vessels were quite normal. No metachromasia was found in the ground substance and PAS staining was negative except for the mast cells (fig. 16).

In guinea pigs submitted to the vitamin C-

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Fig. 12 (Larsen). Large heavily granulated mast cells in a thyroidectomized guinea pig. (Toluidine blue, 0.5 percent, ×1,000.)

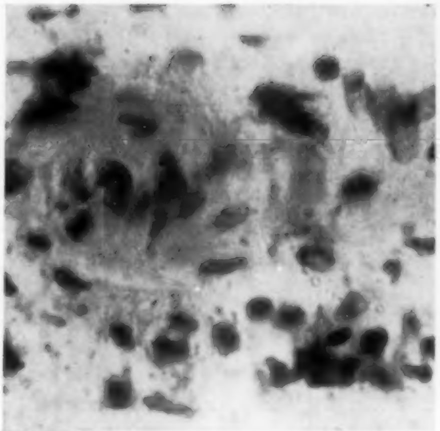

Fig. 15 (Larsen). Thyroxine-affected mast cells in the choroid of a guinea pig. (Toluidine blue, 0.5 percent, ×1,000.)

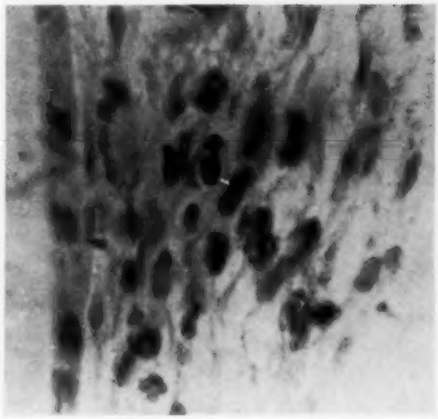

Fig. 17 (Larsen). Degranulated mast cells in the choroid of a scorbutic guinea pig. (Toluidine blue, 0.5 percent, ×400.)

deficient diet, the mast cells responded with heavy degranulation and vacuolization of the cytoplasm in 12 of 14 animals (figs. 17 and 18). The most considerable changes were found in animals kept on the diet for the longest time. Also other cells than the mast cells showed weak metachromatic staining and the intercellular space had increased, giving the appearance of a more immature tissue. With the PAS staining, small areas around some of the cells were slightly pink, the ground substance itself showed no pink color (fig. 19).

The changes of the mast cells described were by far most pronounced in the lead subacetate fixed sections, sections fixed in amino-acridine alcoholic solutions did not reveal the changes in the same degree. Staining with toluidine blue was found to be the method of choice whereas the PAS staining gave variable results. Sections stained with

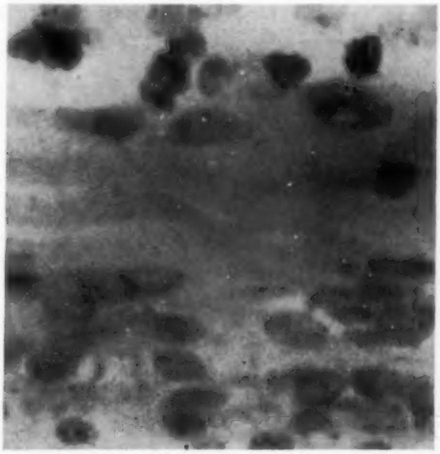

Fig. 16 (Larsen). PAS-positive mast cells in the choroid of a thyroxine-treated guinea pig. (×1,000.)

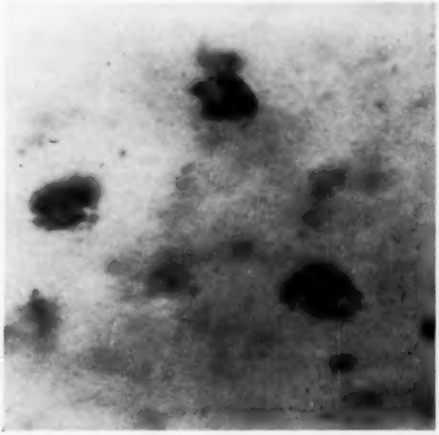

Fig. 18 (Larsen). As Figure 17. (×1,000.)

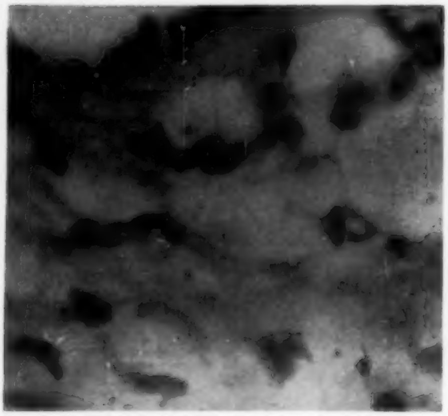

Fig. 19 (Larsen). PAS-positive mast cells and PAS-positive material around other connective tissue cells. (×1,000.)

hematoxylin-eosin were of no value for the evaluation of the changes of the mast cells. In the slides unknown to the author it was found that it was possible to distinguish between characteristic changes of mast cells in 92 percent of the sections.

Discussion

Numerous reports concerning the mast cells have appeared since Ehrlich in 1877[5] found these cells and named them mast cells, but especially after the modern conception of connective tissue as a tissue of pronounced importance, both under normal and pathologic conditions, the mast cells have attracted renewed attention. The mast cells are present in all loose connective tissue, but in varying numbers in different tissues and species of animals.

The cortisone effect on the mast cells has been demonstrated by several authors in histologic sections fixed in various ways (Videbaek, et al., 1950;[6] Asboe-Hansen, 1950;[7] Stuart, 1951;[8] Cavallero and Braccini, 1951;[9] Asboe-Hansen, 1952;[10] Fulton and Maynard, 1953;[11] Buno, 1953;[12] Smith and Lewis, 1954[13] and 1955;[14] Moltke and Zachariae, 1954;[15] Baker, 1953[16]). Wegelius and Asboe-Hansen (1956)[17] have found the same in living, intact connective tissue. Other authors have, however, not been able to demonstrate any change (Schoch and Glick, 1953;[18] Padawer, 1953;[19] Padawer and Gordon, 1953[20] and 1954;[21] Devitt, Pirozynski, and Samuels, 1953[22] and 1954[23]).

In this study the changes after cortisone treatment were found in many of the animals, but by no means in all of them. The effect of cortisone is mostly considered inhibitory of nature because the reduction of the hyaluronidase-sensitive metachromasia has been interpreted as a decrease of the mucopolysaccharides.

Kulonen (1953),[24] however, found an increase of the water binding capacity of the skin following administration of cortisone and investigations of hyaluronic acid by me[25] in rabbit vitreous treated with cortisone revealed increased content of hyaluronic acid. Also, observations in exophthalmos studies of thyroidectomized thyrotrophin-treated guinea pigs have indicated an increase of the water binding capacity of the ground substance following cortisone treatment, probably because of a higher content of mucopolysaccharides (Smelser and Ozanics, 1955[26] and 1958[27]).

Increased numbers of mast cells and higher content of granules in the cytoplasm in thyroidectomized and thyroidectomized thyrotrophin-treated animals have been observed previously (Asboe-Hansen and Iversen, 1951[28]). The response observed in this study was demonstrable in part of the thyroidectomized animals and in nearly all the animals with the combined treatment. The difference might, therefore, be explained as depending on the amount of thyrotrophin present, which seems to be at least one of the provoking factors.

Thyroxine caused degranulation, diminution of the size of the mast cells, especially of the cells some distance from the vessels, in several of the animals. This has also been found by Asboe-Hansen (1950[4]). The effect of thyroxine seems to be an inactivation of the thyrotrophic hormone (Iversen and Asboe-Hansen, 1952[29]). Devitt, et al. (1954[23]),

were not able to confirm these changes after thyroxine treatment in a small number of animals and in alcohol-treated tissue specimens.

In avitaminosis-C the changes of the mast cells were heavy degranulation of the cytoplasm in nearly all the guinea pigs; this has never to the author's knowledge been demonstrated before. The effect of C-avitaminosis seems in some respects to be related to that of cortisone (Ragan, et al., 1949,[30] and Pehrson, 1953[31]). The latter found that in ascorbic acid deficiency there is a comparatively large production of abnormal ground substance which is present in a disaggregated and depolymerized state. This finding has been confirmed by others (Robertson, et al., 1956[32]; Dunphy, et al., 1957[33]).

As borne out from the above the changes of the mast cells after various treatments were by no means uniform, especially in the animals treated with cortisone. It is apparent that the doses were of great importance and also that very high doses were necessary to affect the mast cells and the connective tissue in general. The divergent results obtained by various authors might be explained as a consequence of different doses and also varying techniques, histologic procedures being rife with numerous artefacts, especially the use of alcoholic fixatives seems not very suitable for the purpose; but still this does not fully explain the disagreement in this or other investigations.

The changes of the mast cells found in the groups of guinea pigs submitted to the various treatments at the same time and the tissue sections treated with the same samples of solutions are very difficult to explain as technical variations because the characteristic changes occurred in just the animals receiving the treatment corresponding to the expected changes. Therefore, the difference in response might more readily be explained by our fragmentary knowledge of the mast cells, their origin, and function.

Most authors favor the theory that mast cells are not homoplastic derived cell types, but have their origin from some other connective tissue cells (Michels, 1923[34] and 1935;[35] Balazs, 1953;[36] Bensley, 1952[37]), also their relationship to the basophil leucocytes is unknown. Numerous functions have been ascribed to the mast cells. Only the most recent and more widely accepted will be mentioned.

Holmgren and Wilander (1937[38]) have suggested that the granules of the mast cells were of heparin nature, Sylvén (1941[39]) that the mast cell granules consisted of polysulphuric acid esters, and Asboe-Hansen (1950[40]) that hyaluronic acid is secreted from the mast cells possibly as a heparin-like precursor, and finally Riley, et al. (1953,[41] 1954,[42] and 1955[43]), and others have firmly established that the mast cells are rich in histamine.

Very little is known about the actual mechanism by which the mast cells are affected. Recent investigations by Wegelius and Asboe-Hansen (1956[44]) seem, however, to indicate that the primary change of the mast cells to various stimuli might be a change in the water content of the ground substance; this might again affect the mast cells which respond in various ways to meet the varying conditions in the ground substance depending on which kind of treatment has been induced. If no appreciable change of the amount of tissue fluids occur, the mast cells may remain unaffected, at least morphologically. None of these theories, however, gives a complete understanding of the function of the mast cells. Information pertaining to these cells is, however, accumulating rapidly, but to where it leads is not clear (Padawer, 1954[45]).

Further investigations on the behavior of the mast cells under various pathologic conditions, whether inflammatory allergic, metabolic or degenerative, will probably broaden our understanding in a number of diseases of the uvea.

Summary

Mast cells were studied in the uvea of the eye from various species of animals, follow-

ing administration of hormones and vitamin C-free diet. It was shown that cortisone, thyroidectomy, thyroidectomy plus thyrotrophin, and thyroxin in many, but not in all, of the animals provoked similar changes of the mast cells as published previously by several authors. The changes were to some extent dependent on intensity of treatment, but also unknown factors seemed to be of importance. The mast cells in the scorbutic animals revealed changes very similar to changes following treatment with cortisone.

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AN AUTORADIOGRAPHIC STUDY ON THE UPTAKE OF S^{35}-LABELED SODIUM SULFATE*

IN THE EYES OF NORMAL, SCORBUTIC, AND HORMONAL TREATED GUINEA PIGS

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New York

Several autoradiographic investigations of different tissues in various species of animals have been undertaken, using S^{35}-labeled sodium sulfate as a tracer-substance (Odeblad and Boström, 1952[1]; Boström, Odeblad, and Friberg, 1953[2]; Boström and Odeblad, 1953[3]; Asboe-Hansen, 1953[4]; Jorpes, Odeblad, and Boström, 1953[5]; Boström, review, 1954[6]; Curran and Kennedy, 1955[7]; and others). Recently Dohlman (1957[8]) has reported about the incorporation of S^{35} into the rabbit eye, in which he has examined the time relaionship of the uptake of S^{35} in various parts of the eye and the distribution of radioactive sulfate between inorganic sulfate, ester sulfate, and organic nonsulfated sulfur. In this study autoradiography was used to find out in which tissues and tissue elements the sulfate was incorporated and to demonstrate possible variations in the uptake of radioactive sulfate in the tissues of the

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guinea pig eye under avitaminosis-C, and hormonal treatment. Also the skin of the guinea pig was to some extent examined for comparison.

Material and Methods

Male albino guinea pigs weighing from 350 to 500 gm. were used. Most of the animals (105) were injected with 1.0 mC./kg. weight carrier free $Na_2S^{35}O_4$ intraperitoneally; a small number (17) received, however, 2.0 mC./kg. weight. The animals were killed 24 hours later.

Whole eyes or pieces of eyes (consisting of parts of sclera, cornea, uvea, and retina), and skin were fixed in 0.4-percent 5-amino acridine dissolved in 50-percent alcohol for 24 hours and/or absolute methanol for three hours, dehydrated, and embedded in the paraffin. Some samples were also fixed in 4.0-percent lead subacetate for control purposes. Sections 5.0 µ thick were mounted on gelatine coated slides, and the Doniach and Pelc (1950[9]) technique with fine grain Kodak stripping film was used with a length of exposure varying from 21 to 43 days. Development and fixation of the stripping film were carried out with an amidol developer and 30-percent sodium-thiosulfate for respective four and seven minutes. The sections were stained with 0.5-percent toluidine blue, coated with 2.0-percent elvanol,* Canada-balsam, and cover slip. Sections adjacent to these used for radioautography were stained with the periodic acid-Schiff method and toluidine blue.

The activity in the stripping film was, besides subjective estimation, also evaluated by counting the silver grains in the film over cornea, sclera, choroid, and over the iris and the ciliary body in the groups of animals where the treatment changed the concentration of S^{35}. For this purpose an eyepiece micrometer disc was used. The silver grains in 48 small squares were counted at the magnification of × 1,440, corresponding to an area of 0.005 sq. mm., and the activity of the background subtracted from the total counts. The slides used for counting were carefully selected and the time of exposure was 23 days.

The animals were divided into five groups according to treatment. Twenty-one animals received 15 mg. cortisone daily from five to 14 days; 18 were thyroidectomized and killed three to five weeks later; 19 thyroidectomized guinea pigs were treated with thyrotropin 6.0 U.S.P. daily through five to 10 days; 20 were injected with thyroxine 0.4 mg. daily for seven days; and finally 14 animals survived administration of scorbutic diet for 16 to 20 days.

Results

The radioactive sulfate is taken up by all connective tissues of the eye, leaving other tissues with only very small amounts or traces of activity (fig. 1). In Figures 2 and 3 it is seen that cornea and sclera have a high concentration of radioactive sulfate, about the same level in both. The highest activity is found in the inner layers of these structures where also the highest degree of metachromasia is present. The corneal epithelium shows a low but certain activity, whereas no estimation concerning the uptake of S^{35} of Bowman's and Descemet's membrane is pos-

Fig. 1 (Larsen). Diagram of the distribution of the uptake of radioactive sulfate in the various tissues of the eye. The most intense blackening illustrates the highest uptake, whereas white color represents none or traces of activity.

* "Elvacet" 51-05 polyvinyl alcohol.

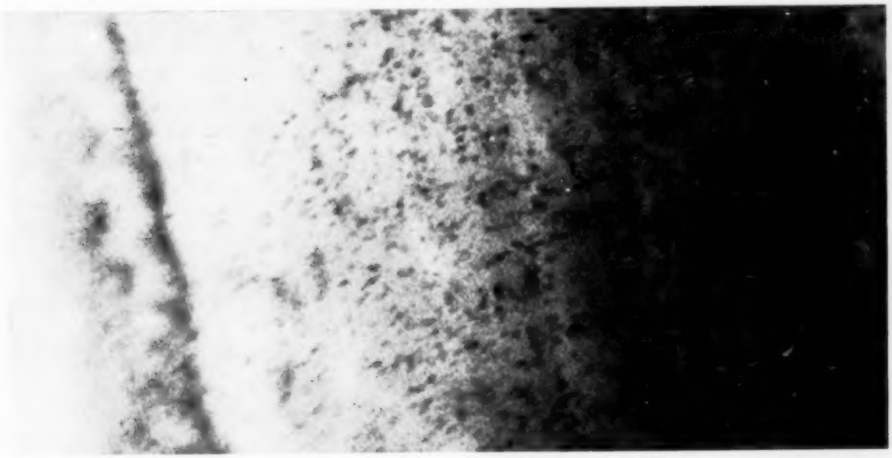


Fig. 2 (Larsen). Autoradiograph* of cornea from a normal guinea pig. Note the high activity in the inner layers and the somewhat lower activity on the outside. A low activity is also seen in the corneal epithelium. (Toluidine blue, ×670.)

* Time of exposure for all autoradiographs: 25 days.

sible because of invariable leaking of activity from the neighboring stroma. The incorporation of radioactive sulfur is by far greater in the intercellular substance than in the stromal cells which only show rather low concentration of activity.

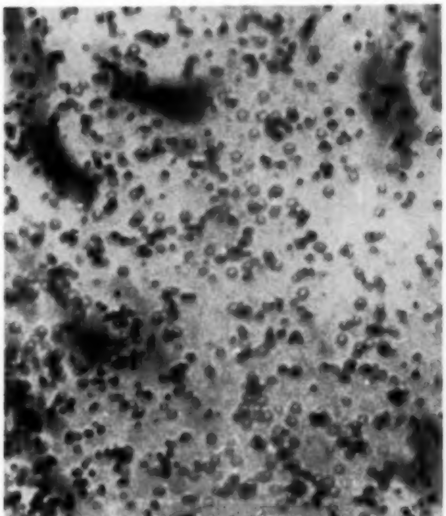


Fig. 3 (Larsen). Autoradiograph of sclera from the same normal animal (as in fig. 2). Note again the high concentration of S^{35} on the inside. The distribution of activity is predominantly extracellular in location. (Toluidine blue, ×1560.)

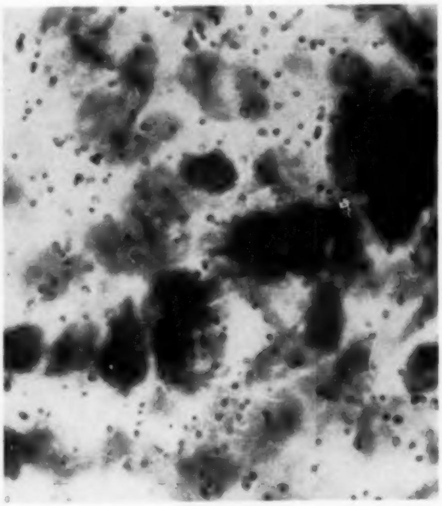


Fig. 4 (Larsen). Autoradiograph of choroid from the same normal animal (as in fig. 2). The radioactivity is low, also predominantly extracellular. No accumulation of activity in any of the cells. (Toluidine blue, ×1560.)

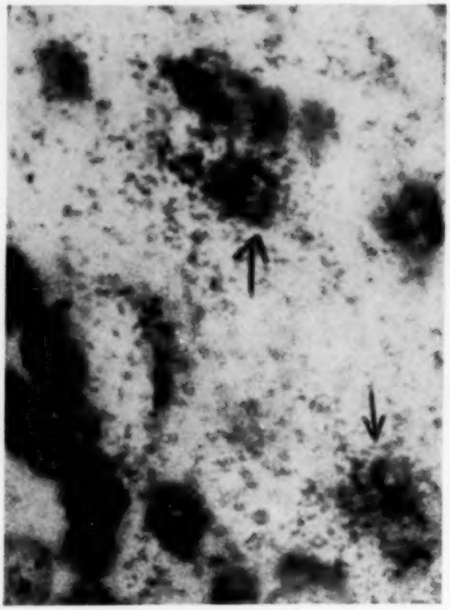


Fig. 5 (Larsen). Autoradiograph of skin in a thyroidectomized thyrotropin treated guinea pig. Intense blackening of the film over two mast cells. (Toluidine blue, ×1560.)

The uptake of S^{35} is considerably smaller in the choroid (fig. 4). No difference, however, was found between the various parts of the uvea. Also in the uvea the content of S^{35} is relatively greater around and between the cells than in the cytoplasm, indicating that the S^{35} is predominantly extracellular in location, neither is an accumulation of S^{35} seen in the mast cells, in spite of the fact that many mast cells are found especially in the choroid. In sections of skin (fig. 5) some but not many of the cells show a high uptake of S^{35} which according to location and shape might be mast cells. The identification of the mast cells in both the uvea and skin was, however, difficult because they failed to stain metachromatic after exposure of the sections to the fixing bath during the developing procedure.

The autoradiograph (fig. 6) indicates that very little to no radioactivity is found in the retina and especially in the pigment epithelium, where an unknown substance which stains like a mucopolysaccharide has been demonstrated with Rinehart's and Abul-Haj

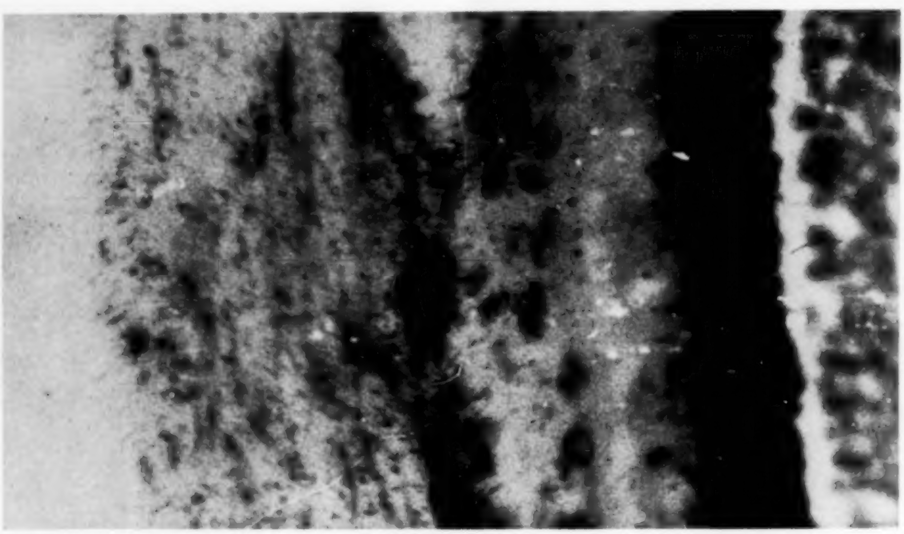


Fig. 6 (Larsen). Autoradiograph of a cross section of a normal eye. No radioactivity in the retina, especially in the pigment epithelium. (Toluidine blue, ×670.)

Fig. 7 (Larsen). Ordinary histologic section of a normal eye with part of the optic nerve. Note the two distinctive dark bands on the two sides of the retina, one corresponding to the pigment epithelium, the other to the most peripheral part of the vitreous body. (Rinehart's stain for mucopolysaccharides, ×164.)

staining technique for mucopolysaccharides (fig. 7).

After treatment with cortisone the uptake of S^{35} was found to be smaller than that in the untreated controls. The difference was, however, not so marked in the cornea as in the other two tissues (figs. 8, 9, and 10).

Still lower activity was encountered in the

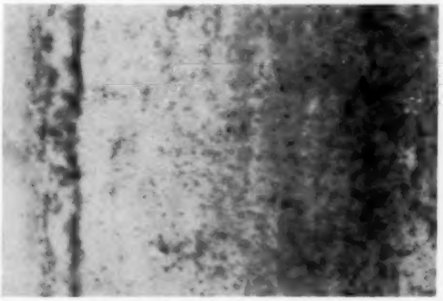


Fig. 8 (Larsen). Autoradiographs of cornea from a cortisone-treated guinea pig, showing slightly less radioactivity than in the normal animal (fig. 2). (Toluidine blue, ×670.)

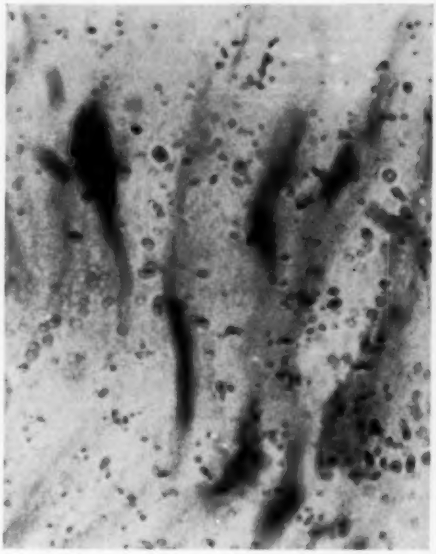


Fig. 9 (Larsen). Autoradiograph of sclera from the same cortisone-treated guinea pig (as in fig. 8), showing distinctively less radioactivity than the control (fig. 3). (Toluidine blue, ×1560.)

autoradiographs from the scorbutic animals (figs. 11, 12, and 13). No evidence of in-

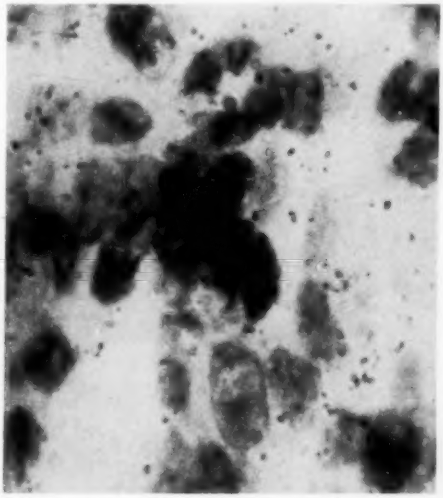


Fig. 10 (Larsen). Autoradiograph of choroid from the same cortisone-treated guinea pig (as in fig. 8), showing smaller incorporation of S^{35}, compared with Figure 4. (Toluidine blue, ×1560.)

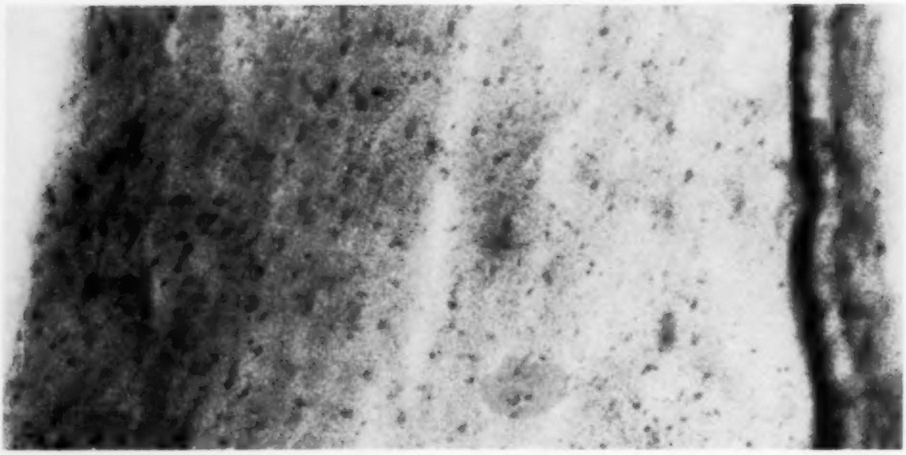


Fig. 11 (Larsen). Autoradiograph of cornea from a scorbutic guinea pig, showing very low activity (Toluidine blue, ×670.)

creased radioactivity was found inside the cells in spite of the fact that many cells, presumably histiocytes, in ordinary histologic sections from scorbutic guinea pigs, stained slightly metachromatic.

The remaining groups (the thyroidectomized, the thyroidectomized plus thyrotropin, and thyroxine treated guinea pigs) revealed no change in the incorporation of S^{35} in any

Fig. 12 (Larsen). Autoradiograph of sclera from the same scorbutic guinea pig (as in fig. 11), showing the same low activity as in cornea. (Toluidine blue, ×1560.)

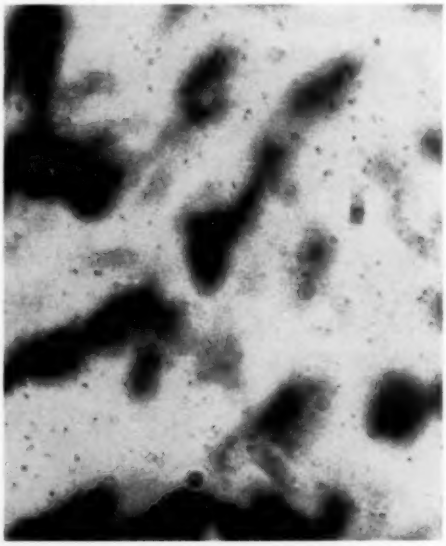


Fig. 13 (Larsen). Autoradiograph of choroid from the same scorbutic guinea pig (as in fig. 11), showing extremely low radioactivity. (Toluidine blue, ×1560.)

TABLE 1

INFLUENCE OF VARIOUS TREATMENTS ON THE ACTIVITY IN THE GUINEA PIG EYE

Treatment	Average Numbers of Silver Grains in Ten Specimens				
	Sclera	Cornea	Iris	Ciliary Body	Choroid
Control	774.9±18.1	805.9±19.7	350.3± 8.2	382.7± 6.5	391.2± 9.3
Cortisone	605.6±27.4	692.6±15.1	278.1±10.3	272.0±12.1	294.5± 8.4
Vitamin-C deficient diet	301.8± 6.5	344.5± 6.9	175.0± 4.7	193.2± 4.7	195.6± 5.0
Thyroidectomy+thyrotropin	797.0±17.0	803.0±16.1	—	—	363.2±10.5
Thyroxine	766.0±20.8	752.0±20.2	—	—	374.8±10.4

of the animals (figs. 14, 15, and 16).

The results of the counting of silver grains are given in Table 1. It is evident that cortisone reduced the radioactivity in a significant degree. In the scorbutic animals the inhibition of the uptake of S^{35} is even greater, whereas no change of radioactivity occurred in the remaining groups.

Comment

From previous studies on the uptake of S^{35} (Borsook, et al., 1937[10]; Dziewiatkowsky, 1949[11]) it is known that the bulk of sulfate is rapidly excreted in the urine. A small fraction is, however, retained in the tissue and incorporated into sulfomucopolysaccharides (Dziewiatkowsky, et al., 1949[12]; Layton, 1951[13]; Boström and Åquist, 1952[14]; Boström and Gardell, 1953[15]). Only an insignificant amount is taken up by other sulfur-containing compounds (Tarver and Schmidt, 1939[16] and 1942[17]; Boström and Aquist, 1952[14]; Dziewiatkowsky, 1954[18]; Chapeville, et al., 1956[19]; Cremer and Dittmann, 1956[20]; Machlin and Pearson, 1956[21]). Most of the inorganic S^{35} is probably removed during the histologic procedure, at least when aqueous fixatives and rinsing of the tissues are used.

As shown previously by Dohlman and Boström (1955[22]) and Dohlman (1957[8]) the highest radioactivity is found in cornea and sclera, where a high content of sulfomucopolysaccharides especially in cornea are present (Meyer, et al., 1953[23]; Polatnick, et al., 1957[24]). In this study no significant difference in concentration of S^{35} was demonstrated between the two tissues, even if the content of mucopolysaccharides, also sulfo-

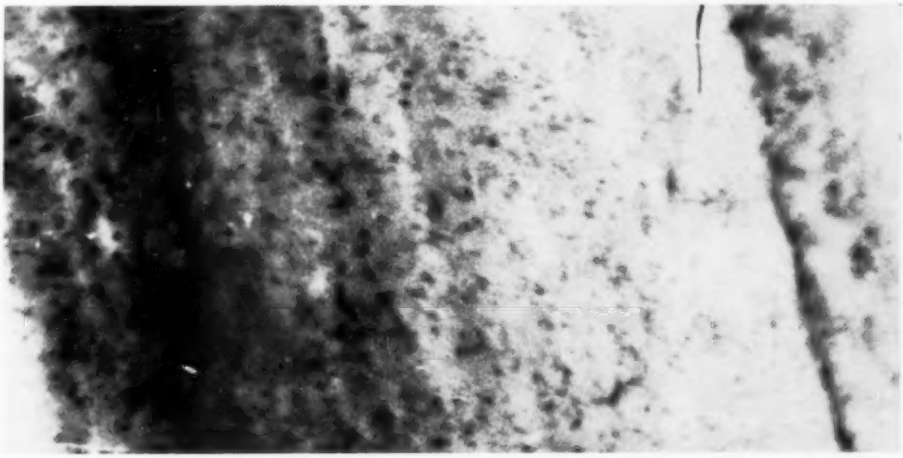


Fig. 14 (Larsen). Autoradiograph of cornea from a thyroidectomized thyrotropin treated guinea pig. The same amount of activity as in the control (fig. 2). (Toluidine blue, ×1670.)

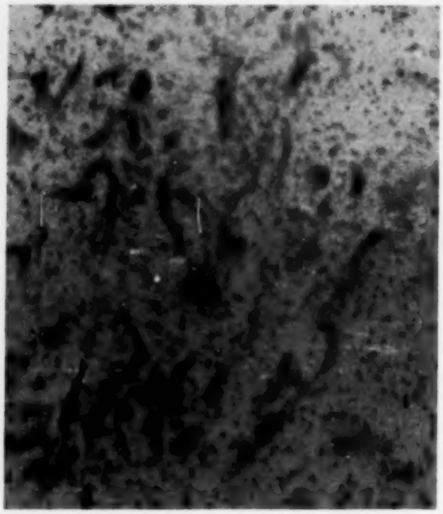


Fig. 15 (Larsen). Autoradiograph of sclera from the same animal (as in fig. 14), showing no change of radioactivity as compared with the control (fig. 3. (Toluidine blue, ×670.)

mucopolysaccharides, is known to be considerably higher in cornea than in sclera.

Dohlman (1957[8]) found a higher radioactivity in sclera than cornea, more than 17 days after the injection of S^{35}. The activity of cornea and sclera is, however, besides concentration of sulfated mucopolysaccharides, dependent on the uptake and turn-over rate of the involved compounds. The uptake of S^{35} is known to be slower and the half-life longer in the cornea than in the sclera (Dohlman and Boström, 1955[22]); sclera seems only to contain chondroitin sulfate B and in smaller amounts A and C (Polatnick, et al., 1957[24]), whereas chondroitin sulfate A, chondroitin, and keratosulfate are found in the cornea. The autoradiographs revealed the highest content of S^{35} in the inner side of these tissues where also the heaviest metachromasia was found.

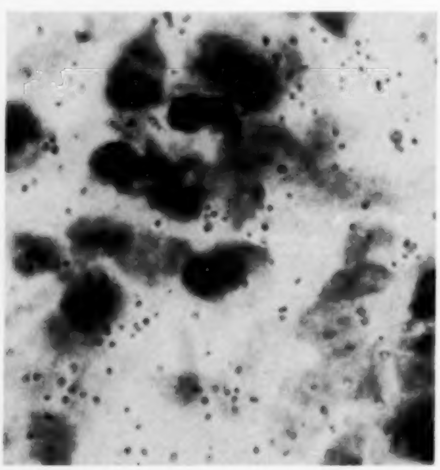


Fig. 16 (Larsen). Autoradiograph of choroid from the same animal (as in fig. 14), showing no change in radioactivity compared with the control (fig. 4.) (Toluidine blue, ×1560.)

Dohlman (1957[8]) has explained this as due to the fact that inorganic sulfate enters cornea and sclera, mainly from the aqueous and choroid and supported this point of view by in vitro experiments, which showed the highest concentration in the outer layers of these tissues (Dohlman and Rodén, 1956[25]). The higher metachromasia, however, points in the direction that there is also a higher content of mucopolysaccharides at this place, with a correspondingly higher S^{35} incorporation.

The lower activity of S^{35} in the uvea was not surprising as the content of mucopolysaccharides presumably is lower than in cornea and sclera. No information is available about the character or the content of the mucopolysaccharides of the uveal tract. Normally, none to vary slight metachromasia is present in the uvea; in thyrotropin stimulated tissue, however, a real metachromasia has been found (Larsen, 1958[26]). Also, here most of the radioactivity was located extracellularly but the concentration of S^{35} was relatively higher in the choroid cells than in the cells of cornea and sclera. The fact that the mast cells in the uvea showed not to take up larger amounts of radioactive sulfate is in disagreement with earlier investigations in other tissues and other species of animals (Asboe-Hansen, 1953;[4] Jorpes, et al., 1953;[5] Curran and Kennedy, 1955[7]) where most of the mast cells showed accumulation of S^{35}.

No studies concerning the uptake of S^{35} in mast cells of guinea pigs have, however, to my knowledge been carried out before.

The reason why the mast cells which are present in large numbers especially in the choroid (Larsen, 1958[26]) did not take up S^{35} in large amounts is unknown. It might be that the metachromatic substance in the mast cells is a non-sulfated mucopolysaccharide or more unlikely that the doses of injected S^{35} were too small, as much larger doses have been used in previous experiments with positive results. Also the state of activity of the mast cells at the time of injection and incorporation of S^{35} may be of importance. Three kinds of nonsulfated mucopolysaccharides are known, namely hyaluronic acid, a heparinlike mucopolysaccharide (Meyer, 1956[27]), and chondroitin. As known, mast cells have been claimed to produce heparin (Holmgren and Wilander, 1937[28]) and hyaluronic acid (Asboe-Hansen, 1950[29]). No conclusion concerning these theories can be drawn from this negative finding.

Cortisone, which is known to reduce the degree of metachromasia and to affect the morphology of the mast cells (Videbaek, et al., 1950[30]; Cavallero and Braccini, 1951[31]; Asboe-Hansen, 1952[32]; Smith and Lewis, 1953[33]; Wegelius and Asboe-Hansen, 1956[34]; Larsen, 1958[26]; and others) decreased the uptake of S^{35} and avitaminosis-C had the same effect in an even higher degree. The inhibitive effect of cortisone has been demonstrated in cartilage in chick embryos previously by Layton (1951[35]), and Boström and Odeblad (1953[36]) in the rat cartilage. Clark and Umbreit (1954[37]) also found an inhibition by cortisone, but an enhancing effect of hydrocortisone on the S^{35} uptake in rats. Other authors (Jones and Gerarde, 1953[38]; Kodicek and Loewi, 1955[39]) have not been able to confirm these findings in experiments in the embryonic chick lung and in rat costal cartilage respectively.

The effect of avitaminosis-C has been shown previously by Reddi and Norström (1954[40]) in costal cartilage, by Upton and Odell (1956[41]) in granulation tissue, and by Moltke (1957[42]) in skin of guinea pigs. Histochemical observations have shown no metachromasia of the ground substance in toluidine blue stained sections (Larsen, 1958[26]) but the intercellular substance has been found to be PAS positive by Pehrson (1953[43]). Nothing is, however, known about the actual mechanism of this inhibition; that is, if it is the uptake of S^{35} or the synthesis of the sulfomucopolysaccharides which is affected.

Meyer (1956[27]) has recently suggested that ascorbic acid might be a precursor to the 1-iduronic acid, the uronic acid chondroitin sulfuric acid B, but further investigations have revealed no evidence for that (Meyer, 1958[44]).

No change in the uptake of S^{35} occurred in thyroidectomized, thyroidectomized plus thyrotropin, and thyroxine treated animals. Earlier studies (Dziewiatkowsky, 1951[45]) have shown no change or a slight decrease of the incorporation of S^{35} in rat cartilage after thyroxine treatment. Moltke 1957[42]) found no significant change in the skin of thyroxine treated guinea pigs, but a decrease of the uptake of S^{35} in wounds. The experiments of Lamberg, et al. (1956[46]), in thyroidectomized, thyrotropin treated guinea pigs showed an increased activity highest 24 to 48 hours after the first injection of thyrotropin. Very little is known about the effect of thyrotropin and thyroxine on sulfomucopolysaccharides, but it seems as if the non-sulfated mucopolysaccharides are predominantly affected by these hormones.

Summary

Autoradiography was used to study the uptake and incorporation of S^{35} in the eye of normal, scorbutic, and hormonal-treated guinea pigs.

A high uptake was seen in the sclera and cornea, highest in the inner layers of these structures, possibly because of a larger content of sulfomucopolysaccharides. The corneal epithelium and endothelium showed a

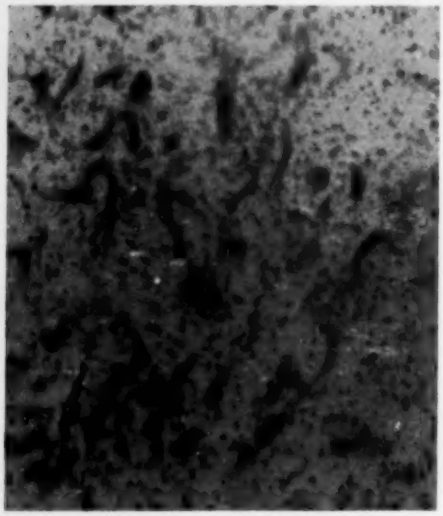
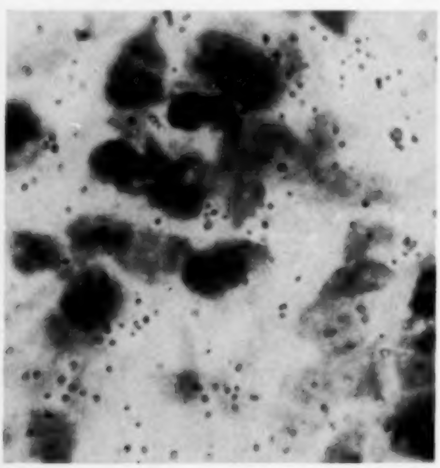


Fig. 15 (Larsen). Autoradiograph of sclera from the same animal (as in fig. 14), showing no change of radioactivity as compared with the control (fig. 3. (Toluidine blue, ×670.)

mucopolysaccharides, is known to be considerably higher in cornea than in sclera.

Dohlman (1957[8]) found a higher radioactivity in sclera than cornea, more than 17 days after the injection of S^{35}. The activity of cornea and sclera is, however, besides concentration of sulfated mucopolysaccharides, dependent on the uptake and turn-over rate of the involved compounds. The uptake of S^{35} is known to be slower and the half-life longer in the cornea than in the sclera (Dohlman and Boström, 1955[22]); sclera seems only to contain chondroitin sulfate B and in smaller amounts A and C (Polatnick, et al., 1957[24]), whereas chondroitin sulfate A, chondroitin, and keratosulfate are found in the cornea. The autoradiographs revealed the highest content of S^{35} in the inner side of these tissues where also the heaviest metachromasia was found.



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A high uptake was seen in the sclera and cornea, highest in the inner layers of these structures, possibly because of a larger content of sulfomucopolysaccharides. The corneal epithelium and endothelium showed a

small, but certain radioactivity. A considerably lower uptake occurred in the uvea. The concentration of S^{35} was found to be predominantly extracellular in location in all the tissues except the corneal epithelium and endothelium. Mast cells in the uvea showed no accumulation of activity. Retina, lens, and the vitreous body revealed only traces of activity.

Treatment with cortisone and vitamin C-deficient diet showed a lower uptake than that of the normal animals, whereas thyroidectomy, thyroidectomy plus thyrotropin, and thyroxine did not change the incorporation of S^{35}.

630 West 168th Street (32).

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Discussion

Frank W. Newell (Chicago): Dr. Larsen has graphically demonstrated by autoradiographs of the guinea pig eye that radioactive sulfur is largely incorporated into the connective tissue of the cornea and of the sclera particularly in the intercellular spaces. Additionally, following the administration of cortisone, there is a lower rate of incorporation into the sclera but the rate is only slightly decreased in the cornea. In scurvy, however, there is a marked decrease of incorporation of sulfur into all tissues. Thyroidectomy, the administration of thyroxin, or thyroidectomy followed by treatment with thyrotropin did not affect the uptake rate of radioactive sulfur. Mast cells in the uvea and in the skin of the guinea pig showed no accumulation of sulfur as has been demonstrated in the mast cells of several other species.

The uptake of radioactive sulfur in the sclera has been shown previously to be at a rate higher than in the cornea. Additionally, both tissues show a higher activity on their inner surfaces suggesting either a greater ionic exchange at these surfaces as compared to the external surface or a greater metabolic activity of sulfonated mucopolysacchrides. The biologic half-life of radioactive sulfur in the sclera of the rat is about 10 days versus about 32 days in the rabbit cornea but this information is not available for the guinea pig. This differ-

BICARBONATE ION IN INTRAOCULAR FLUIDS 531

ence may account for some of the difference in distribution. However, the difference in effect of cortisone and scurvy on the incorporation of radioactive sulfur into the cornea where it has a very slight action and into the sclera where it has a marked effect, suggest that the sulfur is incorporated with different mucopolysaccharides in each tissue. Moreover, a different metabolic mechanism is suggested by the fact that scurvy modifies each tissue uniformly. Dr. Larsen, who has already made important contributions to this subject, has again added to our knowledge concerning these fascinating tissues and their metabolism.

Dr. Godfred Larsen (closing): Concerning the relation between cornea and sclera, it is true, as Dr. Newell pointed out, that the content of mucopolysaccharide, also sulfated mucopolysaccharides in these two tissues, are different. It is much higher in the cornea than in the sclera. Therefore, you would expect much higher uptake in the cornea but until we have more information on the behavior of the various kinds of mucopolysaccharides, we cannot solve these problems. It has been found that there is a difference in uptake and turnover rate. It is much slower in the cornea.

Concerning the different response of cortisone treatment on the cornea and sclera, it may be due to the different kinds of mucopolysaccharides we find here but also the avascularity of the cornea may be of importance.

ELABORATION OF BICARBONATE ION IN INTRAOCULAR FLUIDS*

VI. Rabbit aqueous humor studies with $NaHC^{14}O_3$

Harry Green, Ph.D., and John L. Sawyer, M.D.
Philadelphia, Pennsylvania

The bicarbonate ion in the aqueous humor of the rabbit eye has long been considered to be a product of the secretory activity of the ciliary body, as first postulated by Friedenwald.[1] The subsequent findings of Kinsey[2] that the bicarbonate-ion concentration in the aqueous humor of the rabbit eye was in marked excess over that in the plasma lent experimental support to Friedenwald's postulation and, at the same time, promoted the wide acceptance of the concept that the bicarbonate ion was secreted into the aqueous humor of the posterior chamber by the ciliary epithelium.

The secretory activity of the ciliary body was actually associated with the continuous generation of hydroxyl ions and the immediate precursor of the bicarbonate ion was considered to be CO_2 which was postulated to react with the hydroxyl ions to form bicarbonate ion in the ciliary epithelium. The bicarbonate ion was then considered to diffuse into the posterior chamber, presumably along the concentration gradient from ciliary epithelium to posterior chamber aqueous humor. The CO_2 was formulated as originating from the neutralization of plasma bicarbonate by the hydrogen ions believed to be formed in the stromal area of the ciliary processes.

It was postulated that the net effect of this sequence of movements was the transfer of bicarbonate ion from the blood plasma to the posterior chamber fluid through the intermediary conversion to CO_2 in the stroma and its reformation in the epithelium by reaction with hydroxyl ions. Since the bicarbonate ion concentration in the aqueous humor of the rabbit eye is considerably higher than that in the plasma, the net movement is regarded as one of active transfer of bicarbonate ion.

More recently the participation of carbonic anhydrase was incorporated into the Friedenwald mechanism for the secretion of bicarbonate ion by the ciliary epithelium.[3] According to this the enzyme in the epithelium of the ciliary processes facilitates the neutralization of carbonic acid by hydroxyl ions (reaction 2) by catalyzing the hydration of CO_2 (reaction 1), thereby making H_2CO_3 more readily available. In the stroma, simi-

* From the Research Department, Wills Eye Hospital, and the Department of Biochemistry, Graduate School of Medicine, University of Pennsylvania. This investigation was supported in part by a Fight-for-Sight Grant of the National Council to Combat Blindness, Inc., New York.

larly, the enzyme is considered to facilitate the neutralization of plasma bicarbonate by the hydrogen ions (reaction 3) by catalyzing the dehydration of carbonic acid (reaction 4).

In epithelium:

(1) $$CO_2 + H_2O \rightleftharpoons H_2CO_3$$

(2) $$H_2CO_3 + OH^- \rightleftharpoons HCO_3^- + H_2O$$

In stroma:

(3) $$H^+ + HCO_3^- \rightleftharpoons H_2CO_3$$

(4) $$H_2CO_3 \rightleftharpoons H_2O + CO_2$$

Except for the fact that as the concentration of bicarbonate ion and hydroxyl ions[2] are significantly higher in the aqueous humor of the rabbit eye than in the blood plasma, no direct experimental evidence exists to support the fundamental postulate of Friedenwald that the bicarbonate ion results from the neutralization of CO_2 by hydroxyl ions generated by the oxidation of reduced cytochrome oxidase.*

This investigation was undertaken to determine the source of the bicarbonate ion in the aqueous humor of the rabbit eye by the use of C^{14}-labeled sodium bicarbonate, and of C^{14}-labeled oxidizable substrates, such as pyruvate, succinate, and so forth. This report presents the results of experiments with the intravenous injection of C^{14}-labeled sodium bicarbonate and indicates that the bicarbonate ion in the aqueous humor of the posterior chamber is chiefly derived directly from the plasma bicarbonate.

Procedure

Animals used. Adult pigmented rabbits weighing between four and six lb. were used in all experiments. Each animal was used for only one experiment. During the course of the experiment the rabbit was confined in a wooden box lined with metal with only its head protruding.

Injection of $NaHC^{14}O_3$. Approximately 1.0 mc. of C^{14} as $NaHCO_3$ was injected into the marginal ear vein of the rabbit.

Removal of samples. The aqueous humors were withdrawn first from the posterior chamber and immediately afterward from the anterior chamber of the same eye as previously described.[5] At a later time period the aqueous humors were removed from the opposite eye of the same animal. Blood plasma was prepared from whole blood withdrawn from the ear artery in a heparinized syringe as previously described.[5] Samples of blood were withdrawn at varying time periods.

Analytic

Preparation of material for C^{14} analysis. The "infinitely thick" $BaCO_3$ plate method was employed for counting the radioactivity. The plates containing $BaCO_3$ equal to or greater than 20 mg./cm.^{2} were prepared essentially in accordance with the procedure of Claycomb, et al.,[6] with several modifications regarding the plating assembly. The procedure employed was as follows:

An accurately measured sample (aqueous humor or plasma) was pipetted directly into a glass tissue homogenizer† and then mixed with 64.5 mg. of Na_2CO_3 contained in 0.3 ml. H_2O. The walls of the glass jacket were washed down with approximately 5.0 ml. of H_2O. To the resulting solution warmed to 45 to 55°C.in a H_2O bath was forcefully added an excess of the $BaCl_2$-NH_4Cl solution by means of a hypodermic syringe fitted with a 26-gauge needle. The prior addition of three drops of 10-percent KOH before precipitation of the $BaCO_3$ was sufficient to maintain the pH above 7.0 during the precipitation.

After remaining at 45 to 55°C. for 10 minutes, the contents of the tube was centrifuged in an angle head centrifuge (International PR-1) at 2,000 rpm for 10 minutes. The supernatant was discarded and the precipitate washed twice with H_2O and once with 95-percent ethanol. The $BaCO_3$ residue in the jacket was dried under an infrared

* The correctness of this formulation has been questioned on theoretic grounds.[4]

† The jacket length was shortened to 117 mm. for ease of manipulation in the centrifuge.

lamp. This served to change the $BaCO_3$ into a physical form that could readily be transferred to a planchet with alcohol. Alcohol (1.5 ml.) was added to the cooled contents and the $BaCO_3$ ground by hand into a slurry with a loosely fitting glass pestle, care being taken to disintegrate all clumps. The suspension was then carefully transferred into a tared planchet, the residue in the jacket being washed down with 0.5 ml. of alcohol and transferred to the same planchet.

The final residue may be transferred with a few more drops of alcohol to give better than a 95-percent transfer. However, since the amount of $BaCO_3$ produced was more than that required to give an infinitely thick plate, the total transfer need not be fully quantitative.

The planchet with the radioactive $BaCO_3$ was placed in a round-bottomed brass centrifuge cup (32 mm. × 100 mm.[3]) fitted with a wooden peg (25 mm. diameter) which is approximately eight mm. below the lip of the centrifuge tube. In this way the planchet could be readily and easily removed after centrifugation without disturbing the well-packed residue. The assembly was centrifuged at 2,500 rpm for five minutes and the supernatant removed with a syringe and needle. The planchet and its $BaCO_3$ were dried for about 20 minutes under an infrared lamp and then counted for radioactivity. Each sample was counted for about 12 minutes with a maximum count of 5,000. The accuracy of the counting was within five percent.

Materials and Instruments

$NaHC^{14}O_3$ was obtained from the New England Nuclear Corporation and Tracerlab with a specific activity of 1.0 mc./mMole. Usually 1.0 mc. was contained in 2.0 to 4.0 ml.

The $BaCl_2$-NH_4Cl was 0.5N $BaCl_2$ in 0.4N NH_4Cl. Carrier Na_2CO_3 solution was prepared fresh when needed and contained 64.5 mg. Na_2CO_3/0.30 ml. which gave 120 mg. $BaCO_3$. This when precipitated in a planchet of 4.45 cm.^{2}/area gave 27 mg. of $BaCO_3$/cm.^{2}, sufficient for an "infinitely thick" plate.

Stainless steel cupped planchets, 2.38 cm. diameter and 4.45 cm.^{2} area were obtained from Tracerlab.

For counting radioactivity an El-Tronics Decade Scaler, Model LS1000 AD was connected to an El-Tronics Flow Gas Counter Model FGC-3. As a quench-gas 99.05-percent helium-0.95-percent isobutane was used.

Results

Recovery of radioactivity from plasma. The amount of 0.20 ml. of a standard $NaHC^{14}O_3$ solution was added to 0.20 ml. of plasma and the radioactivity of the resulting infinitely-thick $BaCO_3$ preparation was compared with that prepared from an equal quantity of the standard solution itself. The results of 11 individual replicates in both cases are summarized in Table 1 and show that the radioactivity of the standard sample alone was 22.8-percent higher than that of the standard mixed with plasma. Accordingly, in all experiments the radioactive plates prepared from plasma (0.2 ml. in each case) were corrected by the indicated amount. It is interesting to note that the recovery of C^{14} was independent of the size of the plasma sample from 0.05 to 0.5 ml.

Recovery of radioactivity from aqueous

TABLE 1

RECOVERY OF RADIOACTIVITY FROM PLASMA

To a given volume of solution containing a standard $NaHC^{14}O_3$ was added 0.2 ml. plasma. The activity of the "infinitely thick" $BaCO_3$ plate prepared therefrom, as described under "Analytic" was compared with that from the standard solution itself.

	Standard $NaHC^{14}O_3$	Standard $NaHC^{14}O_3$ +0.2 ml. plasma
No. of replicates	11	11
Average CPM	4,444	3,616
Standard deviation	165.4	90.6
Standard error of mean	49.9	27.3

Correction factor for 0.2 ml. plasma $= \frac{4444}{3616} = 1.228.$

TABLE 2

RECOVERY OF RADIOACTIVITY FROM AQUEOUS HUMOR

Same procedure as in Table 1.

	Standard $NaHC^{14}O_3$	Standard $NaHC^{14}O_3$ +0.2 ml. aqueous humor
No. of replicates	3	4
Average CPM	4,258	4,325
Range	4,124–4,439	4,199–4,390

humors. Comparison of the radioactivity of $BaCO_3$ plates prepared from aqueous humors (0.1 to 0.2 ml. anterior chamber fluid) to which 0.2 ml. of standard $NaHC^{14}O_3$ was added with that prepared from an equal amount of the standard solution itself showed no significant difference between the two (table 2). Therefore, no correction factor for the radioactivity of the aqueous humor samples was necessary.

C^{14} ANALYSES OF PLASMA AND AQUEOUS HUMORS*

PLASMA

The results of 21 experiments are given in Table 3. From a semilog plot of the activity of plasma samples against time it was apparent that the radioactivity disappeared at a single exponential rate. Accordingly, the best straight line was drawn from the plasma data (a minimum of three points) on a semilog plot by graphic methods which meet the criteria of the method of least squares. The slope of each straight line represents the rate of disappearance of C^{14} in the plasma in terms of percent of total C^{14}/minute and the values are listed under the column % Δ/min. While the individual values vary from animal to animal, the average rate of disappearance in the 21 experiments was 6.22 percent/min. This corresponds to an average time for 50-percent disappearance of radioactivity of 11.2 minutes. The time interval during which samples of whole blood was re-

* The following abbreviations have been used in the presentation and discussion of results:

Pl: arterial blood plasma; AC: anterior chamber; PC: posterior chamber; % Δ/min.: percent of the total C^{14} concentration in the given fluid that decreased per minute, or the rate of decrease of radioactivity; CPM: counts per minute; Av: average of values; S.D.: standard deviation; S.E.M.: standard error of the mean; "t": student t-test of significance.

TABLE 3

C^{14} ANALYSES OF PLASMA AND AQUEOUS HUMORS OF ANTERIOR AND POSTERIOR CHAMBERS OF RABBITS FOLLOWING INTRAVENOUS INJECTION OF $NaHC^{14}O_3$

No.	Plasma, %Δ/min.*	Posterior Chamber Fluid				%Δ/min.*	Anterior Chamber Fluid				PC/AC	
		CPM(t_1)	PC/Pl	CPM(t_2)	PC/Pl		CPM(t_1)	AC/Pl	CPM(t_2)	AC/Pl	t_1	t_2
1	5.22	14,780 (11)	2.63	9,650 (20)	2.76	4.73	7,273 (12)	1.35	6,093 (21)	1.82	2.03	1.58
2	6.59	10,300 (11)	1.84	7,310 (20)	2.36	3.82	—	—	5,120 (21)	1.76	—	1.43
3	6.12	12,000 (10)	2.35	5,840 (21)	2.25	6.53	5,341 (11)	1.09	4,021 (22)	1.63	2.25	1.33
4	3.27	16,800 (5)	2.63	9,790 (15.5)	2.15	5.16	5,442 (6.5)	0.89	5,934 (17)	1.37	3.09	1.65
5	10.00	13,620 (6.0)	1.66	7,620 (15.75)	2.46	5.97	5,920 (7)	0.80	5,320 (16.5)	1.86	2.30	1.43
6	5.07	9,370 (11)	1.77	5,515 (20)	1.72	5.90	5,107 (11.5)	1.00	4,698 (20.5)	1.47	1.84	1.17
7	6.57	3,920 (7.25)	1.82	2,950 (15.5)	2.36	3.46	2.819 (7.5)	1.18	2,624 (16)	2.18	1.39	1.12
8	13.80	10,620 (7.5)	2.36	5,220 (16)	3.73	8.33	4,410 (8)	1.05	3,880 (16)	2.77	2.41	1.35
9	7.00	6,950 (8.5)	2.01	2,700 (19.5)	1.69	8.56	2,145 (9)	0.54	1,829 (20)	1.18	3.24	1.48
10	6.66	10,920 (12.5)	2.37	4,448 (25)	2.22	7.20	4,951 (12.5)	1.08	2,954 (25.5)	1.55	2.21	1.51
11	5.94	12,811 (12.5)	1.97	5,638 (24)	1.71	7.12	6,031 (12.5)	0.93	3,559 (24)	1.08	2.12	1.58
12	9.16	4,570 (10)	2.29	—	—	—	1,940 (10)	0.97	—	—	2.36	—
13	6.03	13,329 (10)	1.75	6,249 (25)	2.02	5.07	—	—	—		—	—
14	5.60	—	—	11,879 (14.5)	2.69	—	8,089 (7.75)	1.26	7,785 (14.75)	1.77	—	1.53
15	10.80	15,965 (7.5)	1.45	11,417 (15)	2.33	4.49	6,676 (8.5)	0.67	6,691 (15.5)	1.45	2.39	1.71
16	6.43	10,932 (7.5)	2.60	7,060 (15)	2.72	5.84	5,963 (8.5)	1.76	5,327 (15.5)	2.13	1.83	1.33
17	3.66	—	—	10,464 (14.5)	2.70	—	7,589 (8.25)	1.12	6,952 (15.25)	1.31	—	—
18	5.72	9,464 (7.75)	2.31	6,231 (15.75)	2.40	5.23	3,512 (8.5)	0.90	3,992 (16.0)	1.53	2.70	1.56
19	2.50	—	—	10,550 (18)	2.29	—	2,813 (9.5)	0.49	4,122 (18.5)	0.92	—	2.56
20	2.20	8,516 (7.5)	2.13	6,721 (15.0)	1.98	3.19	4,220 (8.0)	1.06	2,639 (15.25)	0.78	2.02	2.55
21	2.20	8,224 (7.5)	3.02	5,361 (15.0)	2.33	5.67	3,323 (8.0)	1.23	3,417 (15.5)	1.48	2.48	1.57
Av.	6.22		2.16		2.34	5.66		1.022		1.571		
S.D.†	2.82		0.400		0.445	1.50		0.291		0.439		
S.E.M.‡	0.65		0.095		0.099	0.36		0.0686		0.0134		

* %Δ/min.: Percentage decrease of radioactivity concentration per minute.
† S.D.: standard deviation.
‡ S.E.M.: standard error of the mean.

moved from the ear artery for the preparation of plasma was from five to 30 minutes, and usually with not less than eight to 10 minutes between taps.

Posterior chamber

The radioactivity of the aqueous humor of the posterior chamber is recorded in Table 3 as counts/min./0.2 ml. in the columns headed $CPM(t_1)$ and $CPM(t_2)$, the figures in ( ) referring to the time of withdrawal of the fluid. The variable levels of activity observed in each of the two columns are essentially a reflection of the variability of the levels of activity in the plasma, and to the different times of withdrawal of the fluid. More informative and consistent data are obtainable from the ratio of the levels in the posterior chamber to those in the plasma, for corresponding periods of time (PC/Pl). The plasma values for the given experiment were read from the best straight line semilog plots. The average values for the PC/Pl ratios at t_1 and t_2 were 2.16 and 2.34, respectively, and statistically not different from each other. This suggested that the relationship of the activity in the posterior chamber to that in the plasma was essentially constant, at least during the indicated interval of time (five to 25 minutes).

In order to define more precisely the actual relationship between the activities of these two fluids, the individual PC/Pl ratios were plotted against time, as in Figure 1-A. Because of the wide scatter of the experimental points it was difficult to observe a particular trend of the values with time, that is, whether the values rose, fell, or remained constant.

Further resolution of this uncertainty was attempted by calculating the average of values associated with the relatively more dense time groupings, such as between five to 9.5 minutes, 10 to 14.5 minutes, 15 to 19.5 minutes, and 20 to 25 minutes. The results of such calculations are given in Table 4 with the points plotted in Figure 1-B. Here again because of the relatively wide scatter of the individual points, despite the statistical lack of significance beetween them, it was difficult to decide between a straight line curve parallel to the abscissa and a curve with a rise and subsequent fall. Actually when the PC values were plotted relative to the plasma (fig. 4) it was obvious that a straight line with a weighted average ratio of 2.26 best described the relationship between the posterior chamber fluid and the plasma over the indicated experimental time period.

With the establishment of the constancy

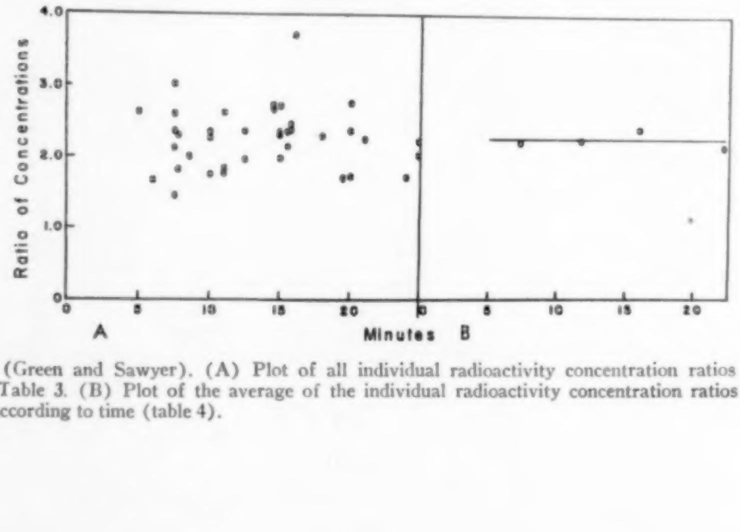

Fig. 1 (Green and Sawyer). (A) Plot of all individual radioactivity concentration ratios (PC/Pl) given in Table 3. (B) Plot of the average of the individual radioactivity concentration ratios (PC/Pl) grouped according to time (table 4).

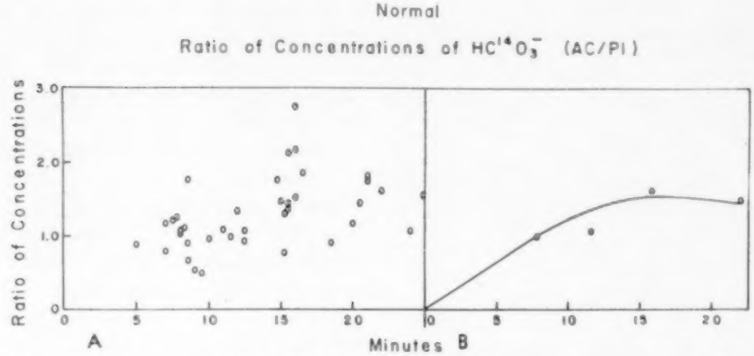

Fig 2 (Green and Sawyer). (A) Plot of all individual radioactivity concentration ratios (AC/Pl) given in Table 3. (B) Plot of the average of the individual radioactivity concentration ratios (AC/Pl) grouped according to time (table 5).

of the PC/Pl ratio with time, it was at once apparent that the radioactivity of the aqueous humor in the posterior chamber must have disappeared at about the same rate as did that in the plasma. That this was the case was confirmed by comparing the average of the individual rates of disappearance (% Δ/min. in table 3). The results showed a value of 5.66 percent/min. for the posterior chamber fluid which was, indeed, not significantly different ($P > 0.40$) from 6.22 percent/min. for the plasma.

Anterior chamber

The radioactivity of the aqueous humor of the anterior chamber is recorded in Table 3 as counts/min./0.2 ml. in the columns headed CPM(t_1) and CPM(t_2), the figures in ( ) referring to the time of withdrawal of the fluid. As with the data of the posterior chamber fluid, here too the variable levels of activity observed in both columns under anterior chamber fluid were essentially a reflection of the variability of the levels of activity in the plasma from animal to animal and to the different times of withdrawal of the fluid. In order to minimize the influence of the individual variability and to achieve more informative and consistent data, the ratios of the activity in the anterior chamber fluid to that in the plasma were calculated (AC/Pl). It can be seen that in every case but one (Experiment 20) the ratio of AC/Pl was higher for the later time period compared to the earlier time period. The over-all averages were 1.022 and 1.571 for t_1 and t_2 respectively.

In order to define more precisely the actual time relationship between the activity in the aqueous humor of the anterior chamber and that in the plasma the individual AC/Pl values were plotted against time, as in Figure 2-A. Despite the relatively wide scatter of the points there was an apparent tendency for the ratio to rise initially and then to fall. Further definition of the trend of the points was achieved by calculating the average of the values associated with the relatively more dense time groupings, such as between five- to 9.5 minutes, 10 to 14.5 minutes, 15 to 19.5 minutes, and 20 to 25 minutes. The results of such calculations are given in Table 5

TABLE 4
RATIO OF CONCENTRATIONS OF RADIOACTIVITY (PC/Pl) GROUPED ACCORDING TO TIME

Time, Min.	No. of Experiments	Average	S.D.*	S.E.M.†	Av. Time, Min.
5–9.5	10	2.199	0.457	0.145	7.25
10–14.5	10	2.236	0.360	0.114	11.70
15–19.5	11	2.403	0.493	0.149	16.00
20–25	7	2.149	0.343	0.130	22.14
Weighted average		2.259			

Discussed under results.
* S.D.: standard deviation.
† S.E.M.: standard error of the mean.

TABLE 5

RATIO OF CONCENTRATIONS OF RADIOACTIVITY (AC/Pl) GROUPED ACCORDING TO TIME

Time, Mins.	No. of Experiments	Average	S.D.*	S.E.M.†	Av. Time, Mins.
5–9.5	13	1.000	0.327	0.0908	7.99
10–14.5	6	1.070	0.141	0.0576	11.60
15–19.5	12	1.629	0.531	0.153	15.80
20–25	7	1.499	0.258	0.0977	22.00

Dicussed under results.
* S.D.: standard deviation.
† S.E.M.: standard error of the mean.

with the points plotted in Figure 2-B.

It is safe to conclude, from the position of the points, that the curve rose initially and reached a peak at 16 minutes or slightly later and then began to fall. It is very significant that after 22 minutes the ratio of activities is 1.499, about the same value as the ratio of the bicarbonate ion concentration in the aqueous humor of the anterior chamber to that in the plasma.[5,7,8,9] Therefore, at longer periods of time, as long as radioactivity persisted in the plasma the AC/Pl value would be expected to have remained constant at about 1.3 to 1.5.

RELATIONSHIP OF RADIOACTIVITY IN AQUEOUS HUMORS OF POSTERIOR AND ANTERIOR CHAMBERS

The ratios of the activity in the aqueous humor of the posterior chamber to that of the anterior chamber for both eyes of each rabbit are given in Table 3 in the last two columns. The t_1 and t_2 refer to the time when each eye was tapped. Since the anterior chamber fluid was removed usually within one minute of the time after the posterior chamber fluid was removed, for the sake of simplicity, the t_1 and t_2 refer to the time of the posterior chamber tap. The individual values are plotted against time in Figure 3-A.

As with the data for PC/Pl and AC/Pl, previously discussed, better definition of the relationship betwen PC and AC activities was obtained from the averages of the values grouped according to time intervals, such as for five to 9.5 minutes, 10 to 14.5 minutes, 15 to 19.5 minutes, and 20 to 25 minutes (table 6). A plot of these average values against the average time of the interval gave, as shown in Figure 3-B, a curve that seemed to decrease exponentially to an asymptote parallel with the abscissa.

A semilog plot of the data, shown in Figure 3-C, gave a good straight line by a graphic method equivalent to the criteria of least squares, thereby confirming the single exponential character of the curve in Figure 3-B. The slope of the curve which indicates the rate at which the ratio decreased was 5.86 percent/min. The exact asymptotic value of the curve could not be determined from the data with any reasonable degree of accuracy. This limitation arises from the fact that it is possible to fit any one of a number of closely similar values for the asymptote without appreciably altering the properties of the earlier part of the curve. Hence, there is no certain criterion for determining which is the best value for the tail of the curve. Suffice to say, nevertheless, that the asymptote should be the same as the ratio of the bicarbonate ion concentrations in the aqueous humor of the posterior chamber to that in the anterior chamber. The average value for this ratio has been reported to be 1.6 to 1.9.[5,8]

DISCUSSION

The rabbits used in this study were considered to be normal and representative of adult pigmented rabbits. As such their physiology and biochemistry were resultants

TABLE 6

RATIO OF CONCENTRATIONS OF RADIOACTIVITY (PC/AC) GROUPED ACCORDING TO TIME

Time, Mins.	No. of Experiments	Average	S.D.*	S.E.M.†	Av. Time, Mins.
5–9.5	10	2.39	0.522	0.165	7.25
10–14.5	8	1.98	0.304	0.150	12.00
15–19.5	11	1.66	0.470	0.142	16.00
20–25	6	1.43	0.150	0.061	21.67

Discussed under results
* S.D.: standard deviation.
† S.E.M.: standard error of the mean.

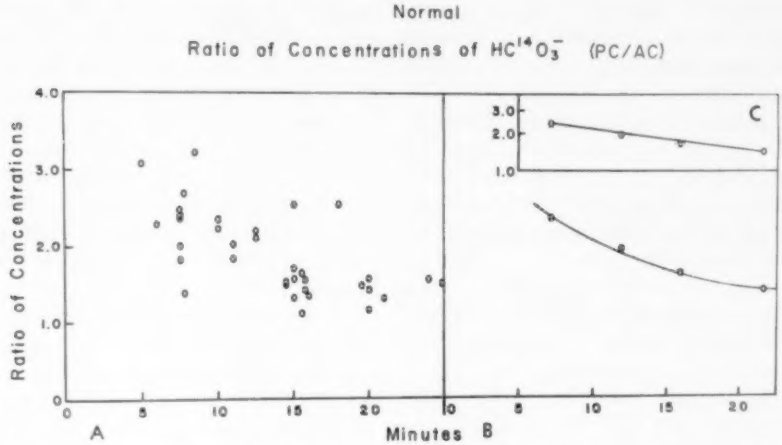

Fig. 3 (Green and Sawyer). (A) Plot of the individual radioactivity concentration ratios (PC/AC) given in Table 3. The time of removal of the AC fluid was taken as the same as that of the PC fluid. (B) Plot of the average of the individual radioactivity concentration ratios (PC/Pl) grouped according to time (table 6). (C) Semilogarithmic plot of data in Figure 3-B.

of normal, steady-state conditions where the rate of removal of the substance studied was equaled by its rates of replacement, so that the amount and concentration of the substance in each compartment or system remained constant during the period of observation. In particular, the steady state conditions of aqueous humor dynamics relative to the bicarbonate ion in this investigation involved the maintenance of an excess concentration of the ion in the aqueous humor relative to that in the plasma, which cannot be explained on a simple physical chemical basis such as the Gibbs-Donnan Membrane Equilibrium. During the time course of the experiment with C^{14}-labeled $NaHCO_3$ the tracer itself was not always in a steady state condition in the three body fluids, but it is reasonably safe to assume that its introduction did not disturb the steady state dynamics of its unlabeled counterpart, either by its relatively small quantity, by the effects of its very soft beta radiations, or by any pharmacologic effects. To be sure the animal is considered not to be able to distinguish the labeled ion from the unlabeled ion, so that normal aqueous humor dynamics can be conveniently studied under normal state conditions by means of the more sensitive and informative tracer technique.

In any investigation of the dynamic relationships of an ion in several body fluids and tissues it is hoped that the experimental data could be subjected to mathematical analysis so as to achieve a quantitative estimate of the desired relationships. In other studies of aqueous humor dynamics[9,10] employing Na^{24} and thiocyanate as test ions, the experimental data were fitted graphically by the compound exponential type of equation describing the elements of ion transfer by secretion and/or diffusion. However, the assumption was made that the concentration of the test substance in the circulating plasma remained constant, in order to simplify the theoretic details, while the graphic methods used in fitting the single or multiple exponential curves were subject to the errors inherent in the customary fitting of straight lines on semilogarithmic charts.[11]

In the current investigation the injected bicarbonate was found to disappear at an exponential rate from which the concentra-

tion of tracer in the circulating plasma at any time could be calculated. It was hoped that the experimental data could be analyzed by exact mathematic procedures[11] and would lead to quantitative estimates of the rates of transfer of the bicarbonate ion into the intraocular fluids. At the same time it was hoped that the results would permit of quantitative estimates of the source of the bicarbonate ion in the intraocular fluids as well as provide experimental insight into the mechanism for its transfer into the eye.

Unfortunately, the accuracy of the experimental data was not sufficiently high either to permit or even warrant exact mathematic treatment. This may be ascribed chiefly to the extreme variability in behavior from animal to animal, as manifested in the data, as well as, presumably, to the significant differences in blood-aqueous barrier permeability between the two eyes of the same animal (see data of table 3). While the magnitude of this variability was too great for an exact mathematic analysis and the calculation of quantitative estimates of transfer rates, the experimental data did permit the description of properties of the so-called "average" rabbit, from which important correlations could be drawn. Each of the curves in Figure 4 represents the calculated average of all the data for the given system.

The blood plasma activity-time curve (—▲—▲—) was described by a single exponential with a rate of disappearance of activity of 6.22 percent/min., the average turnover rate for all the experimental samples (table 3). The component determinants of the disappearance of radioactivity from the blood plasma were essentially the loss of CO_2 through the lungs and exchange of HCO_3^- with the extracellular fluid and tissues. Since the injected radioactive $NaHCO_3$ did not immediately equilibrate with its nonlabeled counterpart, 10 percent being lost via the lungs in the first 30 seconds,[12] the turnover rate of the ion in the first two minutes was undoubtedly faster than during the rest of the time. Because the initial rate of accumulation of the tracer was too fast to be measured, the curve arbitrarily begins at two minutes, the value being the average of all curves extrapolated to this time period.

The posterior chamber aqueous humor activity-time curve was also described by a single exponential with a turnover rate similar to that of the plasma. This is based upon the fact as previously noted under "Results," Figure 1-B and Table 4, that the PC/Pl activities was constant (2.26) during the course of the experiment. It is obvious that the individual PC/Pl values (—o—o—) were not significantly different from those calculated from the average PC/Pl value, (—●—●—) thereby justifying the use of the average.

This constant relationship must signify

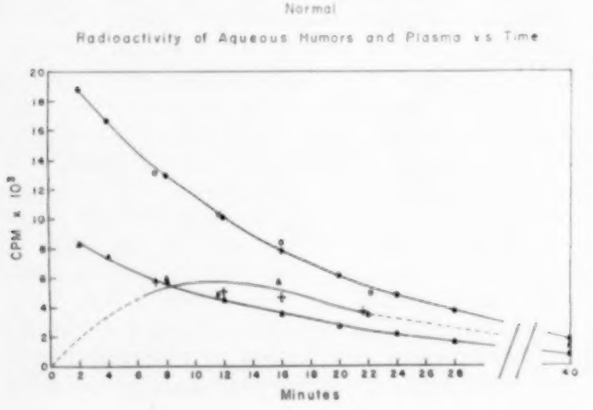

Fig. 4 (Green and Sawyer). Radioactivity concentration of aqueous humors and plasma versus time. The radioactivity concentration is given in terms of counts/min./0.2 ml. All curves are averages as explained in the text under Discussion. Plasma curve, —▲—▲— PC curve, —●—●— (Calculated from average PC/Pl of 2.26) and —O—O— (calculated from individual PC/Pl values grouped according to time); AC curve, —Δ—Δ— (calculated from AC/Pl values grouped according to time) and —+—+— (calculated from PC/AC values grouped according to time).

that the rate of disappearance of the activity in the posterior chamber fluid was essentially the same as the rate of disappearance in the plasma. The PC curve, therefore, describes this constant relationship of the activities in the two body fluids. As with the plasma curve because of the very rapid initial rate of accumulation of the tracer the characteristics of the PC curve during the first two minutes are not certain. To be sure the activity rose from zero at 0 minutes to some maximum value before it began to decrease. Exactly where the peak occurred and the height of the peak are not known. Therefore, the PC curve was drawn arbitrarily starting at two minutes, rather than at 0 minutes.

The anterior chamber aqueous humor activity-time curve was plotted from values calculated from the ratios of AC/Pl (—Δ—Δ—) and PC/AC activities (—+—+) grouped according to time, as indicated under "Results," Figures 2-B and 3-B and Tables 5 and 6. In sketching the AC curve more weight was accorded the eight- and 16-minute points than the 12-minute point, because of the relative number of experimental values upon which the points were based (see tables 5 and 6). The tail of the curve has been extrapolated to 40 minutes to a value 1.25 representing arbitrarily a normal ratio of PC/AC.[5,8,17] (See discussion of fig. 3-B and C under "Results.")

It is immediately apparent that the bicarbonate ion rapidly penetrated the blood aqueous barriers into the posterior chamber within the first two minutes of injection, and, at the same time, established a steady state relationship with the concentration of the labeled ion in the plasma.

As mentioned previously because of the relatively large scatter of the experimental data an exact mathematic analysis of the rates of transfer of the tracer into the eye was precluded. Consequently, the results did not permit any conclusions to be drawn regarding the possible loss of radioactivity from the posterior chamber to the lens and vitreous humor. However, concerning the vitreous humor, in a previous publication[13] evidence was presented that no significant diffusion of bicarbonate occurred from the aqueous humor of the posterior chamber to the vitreous humor.*

In a later report[14] evidence based upon studies with C^{-14}-labeled bicarbonate will be presented to support this finding. As far as the lens is concerned evidence will also be presented in a later report[15] to show that the accumulation of C^{-14}-labeled bicarbonate in the rabbit lens represented only a small fraction of the concentration in the aqueous humor of the posterior chamber. On the other hand, the neutralization of lactic acid from the metabolic activity of the lens may directly account for a small but significant decrease of labeled bicarbonate in the aqueous humor.[16] In view of the probable effect of the lens in using up some of the bicarbonate in the aqueous humor, it is apparent that, even though the rate of decrease of the concentration of radioactivity in the posterior chamber was essentially similar to that of the plasma, it is not completely a reflection of the latter.

The almost immediate attainment of a very significant excess concentration of the labeled ion in the aqueous humor of the posterior chamber relative to that in the blood (average ratio of 2.26) indicated the operation of a mechanism for concentrating the bicarbonate ion in the aqueous humor. If in the isotopic tracer experiment the corresponding ratio of the radioactivities, in the aqueous humor relative to the plasma, under steady state conditions of the labeled bicarbonate, were equal to or greater than the ratio of the unlabeled bicarbonate ions, then one could logically conclude that the major

* In recent experiments, Green and Mann have raised the bicarbonate ion concentration in the posterior chamber aqueous humor by 67 percent by the intravenous infusion of 5.0 and 10-percent $NaHCO_3$. After three hours of the infusion the bicarbonate ion concentrations in various sections of the vitreous humor were not appreciably different from the normal distribution.

bulk, if not all, of the bicarbonate ion in the aqueous humor of the posterior chamber was derived from the bicarbonate ion in the plasma. Similarly, if the ratio of the radioactive concentrations were significantly less than that of the normal steady state distribution of the unlabeled ion, then it would be reasonable to conclude that a significant amount of the bicarbonate ion was derived from another source in addition to that from the plasma.

Under the usual conditions of steady-state in normal rabbit eyes the ratio of the bicarbonate ion concentration in the aqueous humor of the posterior chamber to that in the plasma in our laboratory was about 1.8 to 1.9.[5, 17] Since in the present isotopic tracer experiments the experimentally measured average ratio of radioactivity concentrations was 2.26,* it is reasonable to conclude that the major bulk, if not all, of the bicarbonate ion in the aqueous humor of the posterior chamber was derived from the plasma.

The question naturally arises as to the chemical nature of the substance transferred into the aqueous humor to accumulate as bicarbonate. As discussed in the introduction of this paper the Friedenwald-Kinsey theory of aqueous humor formation was predicated upon the reaction of CO_2 with OH^- in the ciliary epithelium to form bicarbonate ions which then diffused into the aqueous humor of the posterior chamber.

More recently in a discussion of mathematic formulations of aqueous dynamics relative to some experimental data by Green and co-workers,[17] Kinsey[18] underlined the importance of the transfer of CO_2 from the blood to the aqueous humor as representing a substantial source of bicarbonate ion. As Kinsey has indicated the crucial point involved in explaining the dynamics of the bicarbonate system depends upon the demonstration that the CO_2 tension is not the same in all three fluids, that is, the aqueous humors of the anterior and posterior chambers and the plasma. The experimental data used to support this explanation indicated that the CO_2 tension is highest in the plasma and higher in the posterior chamber fluid than in the anterior chamber fluid.

The CO_2 tension is usually calculated from the pH and total CO_2 content or bicarbonate ion concentration of the fluid according to the Henderson-Hasselbalch equation. Small discrepancies in the pH and the CO_2 content of the fluid can give rise to small differences in the final calculated CO_2 tension which become significant when compared with other fluids. Indeed, more recently Langham and Lee[19] reported that the CO_2 tension of arterial plasma (calculated from whole blood analyses) was the same as that of the aqueous humor of the anterior chamber of the rabbit eye.

Examination of the experimental data of Langham and Lee and of Kinsey upon which the conclusions regarding comparative CO_2 tensions were based reveals differences of only 0.03 pH units and 1.0 mM. total CO_2/Kg. H_2O in the blood analyses and 3.9 mM. HCO_3^-/Kg. H_2O in the aqueous humor analyses as the basis for the different estimates. The same considerations apply to calculations of CO_2 tension in the posterior chamber fluid, although Kinsey's estimate is based upon an early measurement[2] of the bicarbonate ion concentration (34.1 mM./Kg. H_2O) of the posterior aqueous.

More recent measurements give values of 39.6 mM. and 38.5 mM. by Becker[8] (averages of 24 and 10 samples, respectively) and 37.0 mM. by Green and co-workers[5] (average of 63 samples). Calculations of CO_2 tension from these values give estimates that show an excess of CO_2 in the posterior chamber fluid compared to that in plasma.

It is, therefore, apparent that the data cited by Kinsey are not unequivocal evidence to support the postulate that the CO_2 tension in the plasma of the rabbit is significantly

* The reason for this relatively high ratio compared to the ratio of 1.8 to 1.9 for the distribution of unlabeled bicarbonate between posterior chamber fluid and plasma is not exactly known.

higher than that in the aqueous humors. Indeed, in view of the recognized ability of CO_2 to equilibrate rapidly across biologic membranes, it is reasonable to assume, in the absence of conclusive evidence to the contrary, that the CO_2 tension of plasma is in equilibrium or very close to equilibrium with that in the aqueous humors.

The foregoing discussion is intended to show that the concept that the contribution of CO_2 to the bicarbonate ion concentration of the aqueous humor may be significant, is not supported by unequivocal evidence.

In attempting to evaluate the significance of the experimental data of the present investigation toward a better understanding of the mechanisms responsible for the elaboration and maintenance of the bicarbonate ion concentration in the aqueous humors, the authors are fully aware of the possibility that their theoretic considerations may not be justified. Nevertheless, in the discussion below and that in a subsequent paper, hypotheses will be advanced which we believe are reasonable and consistent with the experimental data.

Previously it had been concluded from a consideration of the ratio of concentrations of radioactivity in posterior chamber fluid to that in the plasma that the bicarbonate ion in the aqueous humor was derived predominantly from that in the plasma. Obviously, there are essentially two basic considerations relative to the form in which the bicarbonate in the aqueous humor passes from the plasma: (1) conversion of the CO_2, normally in equilibrium with bicarbonate ion, to bicarbonate ion in the ciliary epithelium whence it can diffuse into the aqueous humor of the posterior chamber; (2) the bicarbonate ion as such is the predominant form in which transport occurs into the aqueous humor.

The question now is which of these two basic pathways can best explain the experimentally observed rapid penetration of labeled bicarbonate ion into the aqueous humor of the posterior chamber from the plasma, and the almost equally rapid establishment of the steady state ratio of the concentrations of the labeled ion.

The validity of the first formulation, as descriptive of the pathway for the mechanism of the elaboration of the aqueous humor bicarbonate ion, depends upon the existence of a concentration gradient of CO_2 or CO_2 tension gradient from plasma to aqueous humor, as discussed previously. The fact that the available measurements, calculated from experimental data, indicated that such a gradient probably does not exist, and, in fact, suggested that the CO_2 tension in the posterior aqueous humor may be higher than in the plasma, tends to minimize the importance of CO_2 in the mechanism for the elaboration and maintenance of the relatively high concentration of bicarbonate ion in the aqueous humor. In this connection it is perhaps relevant to the argument against the validity of the $CO_2 \rightarrow HCO_3^-$ mechanism to call attention to the fact that in all considerations of this pathway in the eye the customarily accepted CO_2 tension gradient from venous plasma to arterial plasma has been ignored. Indeed, it is reasonable to assume that the oxidative metabolism of the ciliary processes produces CO_2 within the epithelial cells resulting in a CO_2 tension gradient in the direction of the arterial supply which would tend to oppose the postulated process of the release of CO_2 from the arterial plasma to the cells.

Furthermore, if CO_2 from the blood were the immediate precursor of the HCO_3^- in the aqueous humor, then in the present experiments it would be expected that the C^{14}-labeled CO_2 from the labeled HCO_3^- would be diluted by the nonradioactive CO_2 formed in the cells from the metabolic activity of the ciliary body. The net effect of such a dilution would be a PC/Pl ratio of concentrations of radioactivity significantly less than the normal ratio of 1.8 to 1.9, suggesting that not all of the bicarbonate ion in the aqueous was derived from that in the plasma. The results of this investigation do

not support the theoretic basis for this expectation.

In view of the foregoing considerations it seems reasonable to conclude that the conversion of CO_2 from the plasma to bicarbonate ion in the aqueous humor of the posterior chamber of the rabbit eye probably does not play a significant role in the elaboration or maintenance of the bicarbonate ion concentration in that fluid.

On the basis of the experimental results in the present report it is proposed that the bicarbonate ion of the plasma is transported into the aqueous humor as a bicarbonate ion and that this chemical species is the predominant form in which the transport occurs. It is further suggested that by far the major amount of the bicarbonate ion in the aqueous humor is derived from that in the plasma and that the contribution from other sources (for example, the lens) is relatively small. It is assumed, of course, that the mechanism or mechanisms responsible for this transport are operating so efficiently as to achieve the rapid penetration and accumulation of bicarbonate ion in the aqueous humor.

Regarding the nature of the mechanisms operating to transport the bicarbonate ion into the posterior chamber, the very rapid penetration of the ion is strongly indicative of high permittivity of the blood-aqueous barriers to the bicarbonate ion. This together with the continuous bulk or unidirectional flow of fluid into the posterior chamber from the plasma suggest that part of the bicarbonate ion in the posterior chamber is probably carried in by the influx of fluid from the plasma.* This phenomenon of "solvent drag" has been described by Ussing[22] for the transport of substances across the isolated toad skin. Of course the transport of bicarbonate ion by the fluid cannot account for the establishment and maintenance of the excess in the aqueous humor over that in the plasma. It is, therefore, necessary to postulate that, in addition to entrance by bulk flow, the bicarbonate ion is actively transported† into the posterior chamber, presumably as a result of the metabolic activity of the ciliary body.

Calculation of the relative contribution of the bicarbonate ion entering by solvent drag to the total bicarbonate ion concentration in the posterior chamber fluid depends fundamentally upon two factors:

1. Whether the passage of fluid from the plasma across the ciliary processes occurs primarily through the H_2O-filled intercellular pores or transcellulary.

2. The relative permittivity of the blood-aqueous barriers to the bicarbonate ion.

Regarding the first factor, Ussing[22] observed that the osmotic flow of fluid through the isolated toad skin occurred through the H_2O filled intercellular pores and comprised an essential step in the passage of hydrophilic substances. Similar conclusions were reached by Visscher[23] concerning the osmotic flow of fluid across the intestinal epithelia. Since the available evidence[2] indicates that the bulk flow of fluid into the eye is basically an osmotic flow, it seems reasonable to conclude that this occurs essentially through the H_2O filled intercellular pores of the ciliary processes.

Regarding the relative permittivity of the blood-aqueous barriers to the bicarbonate ion the kinetic data of this investigation, as well as the results of an earlier study,[17] strongly indicate that the bicarbonate ion passes readily from the plasma to the aqueous humor. In view of these considerations it appears

* Grant[21] recently reported that a 6.0 to 8.0 mV. electric potential may exist across the blood-aqueous barriers with the aqueous humor being positive with respect to the blood. Whether this potential difference is sufficient to account for a diffusion of HCO_3^- along a potential gradient remains to be determined. In the meantime by active transport is meant the transfer of the bicarbonate ion from the plasma to the aqueous humor, from a lower to a higher concentration, presumably as a result of the energy derived from metabolic activity of the ciliary body.

† Davson[20] postulated that the diffusible ions were carried into the aqueous predominantly in the primary secretion occurring transcellularly, but not against a concentration gradient.

reasonable to assume that the osmotic flow of fluid from the plasma to the aqueous humor carries or drags with it bicarbonate ion in the same concentration as present in the plasma.

A rough estimate of the relative contribution of these two pathways to the total bicarbonate ion concentration of the posterior chamber fluid may be calculated as follows:

The rate of flow of aqueous humor is assumed to be 1.3 percent of the volume of the anterior chamber (250 μl) of the rabbit eye or 6.5 percent of the volume of the posterior chamber (50 μl) per minute. Therefore, the rate of flow of aqueous humor is 3.25 μl/min. The average concentration of HCO_3^- in the posterior chamber fluid may be taken as 37 mM./l. Therefore, in the 3.25 μl of aqueous humor flowing out of the posterior chamber into the anterior chamber every minute, under steady-state conditions, are 0.1170 μM. HCO_3^-. Similarly in the 3.25 μl of fluid entering the posterior chamber every minute from the plasma with a concentration of 21 mM. HCO_3^-/liter are 0.06825 μM HCO_3^-. The difference of 0.04875 μM. HCO_3^- is the amount of HCO_3^- leaving the posterior chamber every minute in 3.25 μl of fluid which must obviously come from the active transport mechanism. Thus of the total quantity of bicarbonate ion entering the posterior chamber per unit of time 58 percent is presumed to come in with the bulk flow of fluid and 42 precent by an active transport mechanism.

The foregoing calculations were based upon the assumption that the bulk flow of fluid into the posterior chamber is essentially an osmotic flow based upon the relative hypertonicity of the aqueous humor to the plasma. Langham and Lee, however, have pointed out the possibility that the estimates of osmotic pressure of the aqueous humor, relative to the plasma, may be high when measured at temperatures below that of the body. This is based upon the different pH- temperature coefficients for blood and aqueous humor as a consequence of which a decrease in temperature will disturb the two fluids disproportionately. If the bulk flow of fluid into the posterior chamber is not an osmotic flow, then it is necessary to postulate the movement of water transcellularly, either by direct action of the metabolic activity of the ciliary epithelial cells, as a water pump for example, or as a consequence of the active transport of an ion. In any event, it is quite probable that the flow of fluid will carry with it bicarbonate ion. If the cell tends to concentrate bicarbonate within it, then the transcellular movement of H_2O may be responsible for a greater proportion of the total bicarbonate ion concentration in the posterior chamber than calculated above.

On the other hand the loss of bicarbonate from the aqueous humor to the lens, probably by diffusion together with the neutralization of bicarbonate by lactic acid from lens metabolism, suggest that the contribution of the active transfer mechanism is probably higher than the 42 percent estimated. Therefore, in view of the absence of exact knowledge of the extent of these factors, it is understood that the above calculations are only approximate estimates of the relative contributions of the two postulated pathways to the elaboration and maintenance of the bicarbonate ion in the aqueous humor of the posterior chamber.

The data of the present investigation do not permit any definite conclusions to be drawn regarding a relationship between the active transport of the bicarbonate ion and the hypertonicity of the aqueous humor which is presumably the driving force for the net influx of fluid into the posterior chamber.

Regarding the kinetics of radioactivity in the aqueous humor of the anterior chamber, the curve drawn in Figure 4 indicated that the rate of accumulation was much slower than that in the aqueous humor of the posterior chamber. After about eight minutes the radioactivity in the aqueous humor was equal to that in the plasma, and, subsequently,

as the concentration continued to fall in the plasma, the concentration in the anterior chamber continued to rise, reaching a peak in about 16 minutes. During the next 10 minutes, or so, a steady-state relationship with the plasma was reached and maintained, presumably, for an indefinite period of time. While the concentration of radioactivity in the anterior chamber aqueous humor reached a level higher than that in the plasma, it always remained below that in the posterior chamber fluid. Although it is presumed that the bicarbonate ion enters the anterior chamber both by flow from the posterior chamber and by diffusion from the plasma, the results of the present investigation do not permit any accurate calculations regarding the comparative rates of penetration of labeled bicarbonate from these two sources.

Another approach to our understanding of the dynamics of aqueous humor of the anterior chamber and its chemistry is based upon recent measurements[24] of osmotic pressure in this laboratory by a new cryoscopic method which indicate that the aqueous humor of the anterior chamber is hypertonic to that in the posterior chamber. On the basis of vapor pressure studies Kinsey[25] concluded that the osmotic pressures of the two aqueous humors were the same, suggesting that no significant net transfer of H_2O occurred across the blood aqueous barriers of anterior chamber. It was, therefore, reasonable to explain the lower bicarbonate ion concentration in the aqueous humor of the anterior chamber compared to the concentration in the posterior chamber as resulting from a net diffusional loss to the plasma.

Davson and Luck[26] suggested that the most probable explanation is the formation of lactic acid by the lens and cornea. However, the recently measured hypertonicity of the anterior chamber fluid, not only with respect to the plasma but also relative to the posterior chamber fluid implies a significant osmotic flow of fluid into the anterior chamber from the plasma. This net influx of fluid is considered to drag with it bicarbonate ion in the same concentration as present in the plasma.

Since the latter is markedly lower than the concentration of bicarbonate ion flowing into the anterior chamber from the posterior chamber, the net result would be a lower bicarbonate ion concentration in the anterior chamber compared to that in the posterior chamber fluid. What effect such a bulk flow of fluid from the plasma into the anterior chamber would have upon the concentration of other substances entering from the posterior chamber would depend entirely upon the relative permittivity of the blood aqueous barriers of the anterior chamber to the substance, and upon its concentration in the plasma relative to that in the aqueous humor.*

Naturally, the existence of an excess osmotic pressure in the anterior chamber over that in the posterior chamber raises questions regarding the source of this hypertonicity and whether one or more specific ions may be actively transported into the anterior chamber to account for this osmotic influx of fluid. The answers to these as well as to questions as to the rate of influx of the fluid into the anterior chamber from the plasma must await further experimental data.

Summary

1. The kinetics of penetration and accumulation of bicarbonate ion in plasma and aqueous humors of the rabbit were studied with the use of C^{14}-labeled $NaHCO_3$ injected intravenously.

2. The tracer in the plasma was found to decrease at an exponential rate, so that 50 percent of the radioactivity disappeared in 11.2 minutes.

3. The bicarbonate ion penetrated the posterior chamber very rapidly within the first two minutes and the concentration decreased at the same rate as that in the plasma.

4. The bicarbonate ion penetrated the anterior chamber more slowly and the concen-

* We wish to express our sincere appreciation to Dr. Samuel I. Askovitz for his valuable discussion and helpful mathematic suggestions and to Dr. Irving H. Leopold for his encouragement.

tration reached a steady relationship with that in the plasma and in the posterior chamber in about 20 minutes.

5. While the concentration of tracer in the posterior chamber aqueous humor was two times that in the plasma, at least after about two minutes, the concentration in the anterior chamber became equal to that in the plasma after about eight minutes and exceeded the latter by some 50 percent in 16 minutes, after which time the concentration slowly fell at about the same rate as did that in the plasma.

6. On the basis of the available evidence and theoretic considerations it has been suggested that the conversion of CO_2 to bicarbonate does not contribute appreciably to the bicarbonate concentration in the aqueous humors.

7. It is suggested that the major bulk of the bicarbonate ion in the aqueous humor of the posterior chamber is derived directly from the bicarbonate ion in the plasma and that this is the chemical form in which it penetrates the eye.

8. The bicarbonate ion is considered to enter the aqueous humor of the posterior chamber by two mechanisms: (1) an active transport mechanism, presumably dependent upon the metabolic activity of the ciliary body; and (2) the passive mechanism of being carried in by the bulk flow of fluid.

Smith, Kline and French Laboratories (1).

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Discussion

Dr. V. Everett Kinsey (Detroit): There are several points on which we disagree with the authors. However, let me open the discussion by showing the point of agreement. This concerns the data themselves.

(Slide) I show this merely to show that we obtained results which are quite similar to those presented except the ratio of concentration of aqueous to plasma at steady state is more nearly that found by chemical analyses in our experiments.

The important point, as Dr. Green mentioned, is the vast difference between the turnover rate of bicarbonate and that of other substances, with the possible exception of water. Bicarbonate obviously is turning over more rapidly than anything that has been measured, including water. This brings up the point upon which Dr. Green and I disagree.

As you all know, carbon dioxide and bicarbonate exist together in the blood—the ratio of concentration being pH dependent. In the case of the aqueous humor, about three and one-third percent of the total bicarbonate is in the form of carbon dioxide, and in the plasma about five percent. Thus, while labeled bicarbonate ion is injected, in a matter of seconds a fixed portion of the labeled carbon will exist as C^{14}, we believe, diffuses from the plasma through the ciliary epithelium into the posterior chamber. Perhaps in the ciliary epithelium, the radioactive CO_2 exchanges with the unlabeled carbon of the bicarbonate ion. This exchange occurs rapidly also.

If one postulates (as we do) that the bicarbonate ion itself goes much slower than the CO_2—something like sodium—then the CO_2 going in as the labeled carbon can exchange rapidly with bicarbonate ion; and become trapped since it cannot get out nearly as rapidly as it entered in the form of free CO_2

I do not believe, in other words, that the experiments indicate the rate of turnover of bicarbonate ion.

One must remember that these are tracer experiments. This to and fro movement does not require a concentration gradient. Dr. Green states in his paper that I believe there is a concentration gradient of CO_2 between plasma and anterior chamber aqueous. There are some weaknesses to these data as Dr. Green points out. However, so far as the present experiments are concerned, it is not necessary for the rapid exchange of bicarbonate that there be any gradient whatsoever; in fact, the gradient could be negative, that is, more CO_2 in the posterior chamber than in the plasma, and still there would be a very rapid exchange.

Dr. Harry Green (closing): I would like to express my appreciation to Dr. Kinsey for his kind remarks and for stimulating the discussion with his provocative comments. This phase of aqueous humor studies will continue to be controversial until, in my opinion, definitive experimental data become available which will permit an unambiguous and generally acceptable understanding of the mechanism whereby the bicarbonate ion enters the aqueous humor.

In the meantime I submit that the concept herein introduced is consistent with the reported experimental data and can explain the dynamics of the bicarbonate ion in the aqueous humors of the rabbit eyes just as adequately as any other theory. As long as there exists more than one explanation of the available experimental results, then it behooves us as scientists to give consideration to each.

It is not to be construed, from the data presented, that we can precisely describe the mechanism whereby the bicarbonate ion is maintained in excess in the rabbit aqeuous humor. This remains to be elucidated. Perhaps results to be reported at a later date describing the effect of Diamox upon the turnover rates of C^{14}-labeled $NaHCO_3$ will shed some light upon this. Suffice to say, for the present, that Diamox does indeed lower the rate with which the bicarbonate enters the aqueous humor from the circulation.

OUTFLOW PATTERNS OF THE CAT EYE*

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Grant,[10,11] by means of tonography, and Goldmann,[8,9] by using anterior chamber fluorescein turnover rates, determined the resistance to aqueous outflow and its reciprocal, the facility of outflow (C). The values of C, by Goldmann's method, applied to intraocular pressure levels between the normal and the episcleral venous pressure. By the method of Grant the C values referred to the pressure range between that induced by the weight of the tonometer and the normal intraocular pressure. Both methods have their limitation as emphasized by Becker,[4] Goldmann,[7] Ballintine,[1] and others. Subsequent perfusion experiments[2,3,5,6,12] verified the results of these investigators and indicated that C was constant over much wider ranges of pressure.

In more recent studies on the distensibility of the intact cat[13] and human[14] eye, pressure-volume relationships were used to express the function of elasticity. Here, fixed volumes of fluid were injected into the anterior chamber and the pressure changes recorded. From the readings, a curve could be constructed which related volume changes to pressure. This type of interpedendence lent itself well to the manometric estimation of outflow. The intraocular pressure was elevated, by infusion, to any desired pressure; the fall of pressure per unit time was converted to volume change, expressed in cmm./min. The volume outflow per minute was plotted against the mean intraocular pressure for the specific flow interval. The slope of the plot then becomes the expression of the facility of outflow.

In the course of studying the effects of various pharmacologic agents and nervous influences on the facility of outflow by this method, three types of outflow patterns became apparent. Two of these occurred regularly; the third could be induced by the administration of hypotensive agents.

Methods

Young adult cats weighing between 1.8 to 3.2 kg. were used in these experiments. The animals were anesthetized with pentobarbital sodium, 35 mg./kg., administered intraperitoneally.

The femoral artery and vein of one leg were cannulated with polyethylene tubing. The femoral artery cannula was connected to a Sanborn Electromanometer and Polyviso recorder for the registration of blood pressure. The venous cannula was used for the administration of drugs and supplemental anesthesia. A cannula was placed in the trachea so that artificial respiration could be administered whenever desired. Two 27-gauge needles attached to 5.0 cm. of No. 10 polyethylene tubing were inserted into the anterior chamber at the limbus. One of these needles was used for recording of eye pressure and the other for infusing fixed volumes of isotonic saline, as previously described.[13]

The eye pressure changes were measured by means of Statham transducers (Model P23BB) or by Sanborn transducers (Model 267B). The volume displacement of the Statham transducer is listed by the manufacturer as 13 cmm./100 mm. Hg. The Sanborn transducer has a stated displacement of 0.03 cmm./100 mm. Hg. The intraocular pressures were recorded synchronously with the blood pressure.

The outflow patterns were determined in the following manner: the eye pressure was elevated, by infusions of saline, to levels approximating 80 mm. Hg, and then allowed to fall spontaneously. The intraocular pres-

* From the Ophthalmology Branch, National Institute of Neurological Diseases and Blindness, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

sure was read generally at 30-second intervals during the entire period of fall. The pressure readings were converted to volume units by means of a pressure-volume curve previously ascertained for each eye. The volume change expressed in cmm./mm. was plotted versus the mean intraocular pressure for the specific time interval. The slope of the curve thus obtained is C or outflow/min./mm. Hg. Measurements were considered unacceptable if changes in blood pressure exceeded 5.0 mm. Hg during the determination period.

Results

Monophasic outflow patterns

In 72 determinations of facility of outflow (C) on 14 eyes, patterns were obtained which resembled those reported by Grant.[10-12] The fall of the previously elevated eye pressure as a function of time for this type outflow is shown in Figure 1. The calculated outflow per minute is directly related to the intraocular pressure (fig. 2). As pointed out by Becker,[2] the pressure intercept of such curves represents the equilibrium pressure within the eye. In these experiments the intercepts were found to be ±4.0 mm. Hg of the known equilibrium pressures recorded

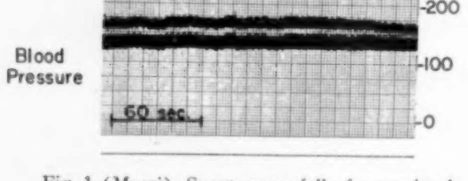

Fig. 1 (Macri). Spontaneous fall of a previously elevated eye pressure. Monophasic outflow pattern. The intraocular pressure was raised by infusion of saline into the anterior chamber and was then allowed to fall spontaneously.

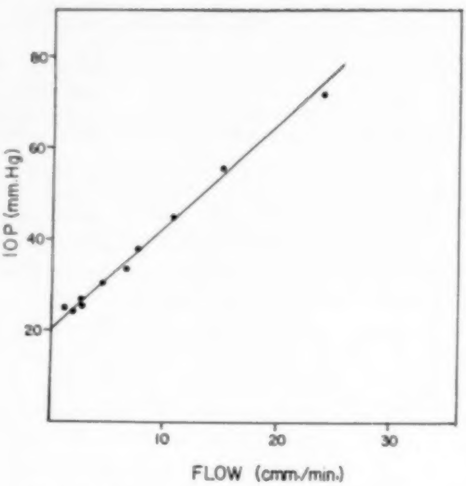

Fig. 2 (Macri). Monophasic outflow pattern. Outflow can be seen to bear a direct linear relationship with the intraocular pressure.

prior to each determination. The mean value of C in this group of experiments was 0.28 ± 0.12, with extremes of 0.08 to 0.65.

Biphasic outflow patterns

Sixty-three determinations of C in eight eyes produced outflow curves which were not a single straight line function of the intraocular pressure. The rate of fall of the previously elevated intraocular pressure for this type outflow is seen in Figure 3. Plots of outflow per minute versus the intraocular pressure gave curves which, in most cases, could be construed to be biphasic (fig. 4). The first limb of the curve obtained at the lower intraocular pressures had values of C which were always greater than those of the second limb, observed at the more elevated pressures. C of the lower limb averaged 1.8 cmm./min./mm. Hg; that of the upper limb averaged 0.35 cmm./min./mm. Hg. The lower limb of the outflow curves generally intercepted the pressure axis at points within ±3.0 mm. Hg of the known equilibrium starting pressure.

This outflow curve was also plotted as flow/minute versus the log pressure (fig. 5).

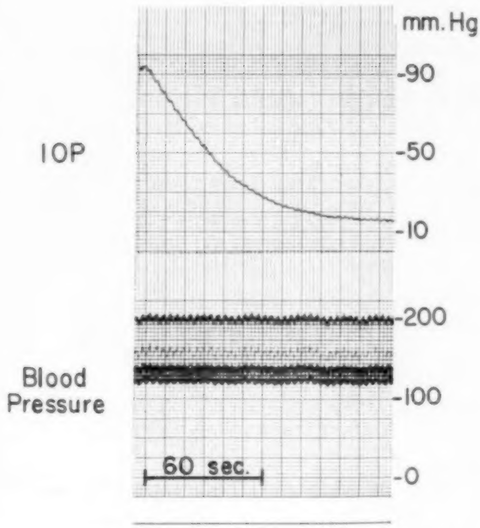

Fig. 3 (Macri). Spontaneous fall of a previously elevated eye pressure. Biphasic outflow pattern. The intraocular pressure was raised by infusion of saline into the anterior chamber and was then allowed to fall spontaneously. The intraocular pressure can be seen to decline more rapidly than that of the monophasic outflow of Figure 1.

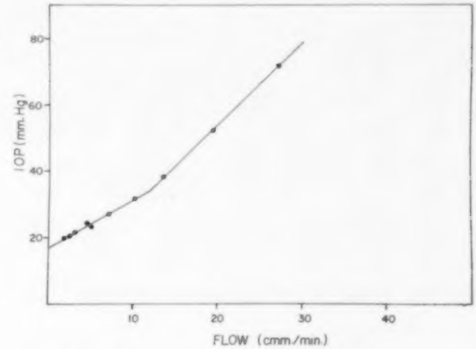

Fig. 4 (Macri). Biphasic outflow pattern. Lines could be fitted through the points of this type of outflow curve to indicate two distinct rates of flow. The rates obtained at the higher pressure levels were always less than those obtained at pressure values approaching the normal. A straight line relationship of flow to pressure could generally be obtained if the points were plotted versus log pressure (see fig. 5).

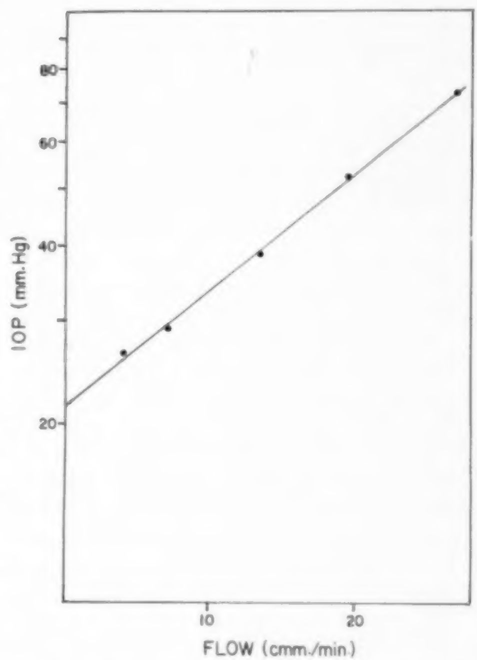

Fig. 5 (Macri). Biphasic outflow pattern. Plotting the points of Figure 4 as outflow/minute versus log pressure generally produced straight line curves.

A straight line function was usually obtained with the intercept falling within 1.0 to 3.0 mm. Hg of the known equilibrium pressure. In current experiments, the biphasic outflow pattern has also been found to occur in enucleated eyes.

Polyphasic outflow pattern

A polyphasic outflow pattern could be induced experimentally and was also seen to occur spontaneously in animals whose blood pressures were around 125 mm. Hg and less. The response was brought about by the intravenous administration of prototypes of the various classes of hypotensive agents: hexamethonium Br, hydrazino phthalazine, veratrum veride, and tolazoline. Doses of these agents, which were sufficient to produce sustained falls of blood pressure, evoked this type of outflow pattern. The eye pressure fall observed under these conditions is seen in Figure 6. The intraocular pressure falls rapidly at eye pressure levels

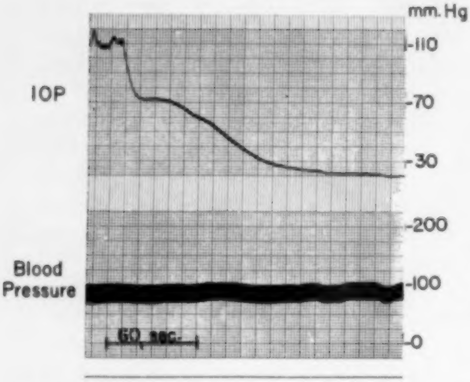

Fig. 6 (Macri). Spontaneous fall of a previously elevated eye pressure. Polyphasic outflow pattern. The intraocular pressure was raised by infusion of saline into the anterior chamber and was then allowed to fall spontaneously. The oscillations noted in the eye pressure tracing at the high pressure level were due to the infusion device. The intraocular pressure can be seen initially to fall very sharply. After remaining relatively constant for 20 seconds, the pressure again continues to decline to base levels. The animal was administered hexamethonium Br[+], 2.0 mg./kg., intravenously, 15 minutes prior to this recording.

greater than the experimentally lowered systemic blood pressure. At pressures approximating that of the blood pressure, the decline is less steep and the cardiac waveforms appear in the eye pressure tracings. The intraocular pressure then assumes a relatively constant value for 10 to 30 seconds and proceeds to fall again to its normal values.

A triphasic outflow pattern is obtained (fig. 7) when flow/minute is plotted against the intraocular pressure. The uppermost limb of the outflow tracing decreases very rapidly to intercept the pressure axis at levels which are slightly below that of the systemic blood pressure. The curve inflects and flow rate increases as the eye pressure falls further. A second inflection point is seen at pressures between 40 and 60 mm. Hg. From this point the rate of outflow decreases until the normal ranges of eye pressure are reached. This outflow pattern has not yet been found to occur in either enucleated human or cat eyes.

Discussion

The present report indicates the feasibility of manometric determinations for "facility of outflow" in animals. The procedure is not difficult and does not consume more time than tonography, after the preparation of the pressure-volume curves. Similar to the techniques of perfusion, the method requires no consideration of elasticity changes of the eye.

The measurements reported here represent total volume changes of the eye, presumably due to aqueous outflow. There is the distinct possibility, however, that the reported data also reflect, to a certain degree, an effect of compression and refilling of the vascular bed, particularly at the higher intraocular pressure levels.

In the present series, three patterns of outflow were observed. The first is similar to that reported by Grant: outflow per minute is proportional to the intraocular pressure over

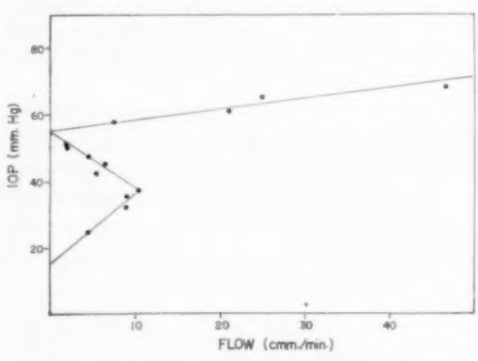

Fig. 7 (Macri). Polyphasic outflow pattern. This determination was made two minutes after the administration of hexamethonium Br, 2.0 mg./kg., intravenously. The mean systemic blood pressure was 61.0 mm. Hg. The decrease of ocular volume, expressed as outflow, declines very rapidly (upper limb) to intercept the pressure axis at a level close to that of the mean systemic blood pressure. Outflow then increases (middle limb) as the eye pressures fall to levels usually between 40 and 60 mm. Hg, in this illustration to 38 mm. Hg. At this point the curve inflects (lower limb) and indicates a decreasing outflow with further falls of pressure until base levels of eye pressure are reached. The lower limb of the curve may well be the only segment of the curve which is representative of the true outflow.

the entire range studied (app. 80 mm. Hg to base levels). This has been referred to as a monophasic outflow pattern. The mean value of C obtained in intact living eye was found to be 0.28. The curve intercepts the pressure axis at levels within ±4.0 mm. Hg of the known base pressure.

The second pattern is considered as biphasic. The outflow per minute is disproportionate to the intraocular pressure. The facility of outflow increases progressively as the intraocular pressure falls from the elevated to the normal intraocular pressure level. On many occasions, two intercepting straight lines could be drawn through the plotted points to express outflow as a function of pressure. The biphasic curves are also amenable to plotting by using the logarithm of the intraocular pressure and give usually a single straight line curve. It appears then that under certain conditions aqueous outflow bears a linear relationship to pressure with two different rates of "facility of outflow," or outflow may be considered to be linear with the logarithm of the pressure.

The third type of outflow pattern noted, which is called polyphasic, is best explained on a basis of vascular changes in the eye. At eye pressure levels greater than the experimentally lowered systemic blood pressure, collapse of the intraocular vessels must occur. As the intraocular pressure falls to a level approaching that of the intraocular arteries, the vascular tree beings to refill. When the rate of outflow of aqueous equals the rate of vascular refilling, no pressure or ocular volume change will occur. This situation is represented by the uppermost limb of the triphasic outflow curve. After a few seconds of equilibration between aqueous outflow and blood volume influx, aqueous begins to flow out of the eye at rates greater than the additional inflow of blood. A point is finally reached where the blood vessels reach their maximum fullness and no longer influence the volume of the eye. This is represented by the second segment of the outflow curve. In the lowermost limb it is presumed that the blood volume changes no longer mask aqueous outflow so that this segment of the curve is most representative of the true outflow.

Summary

Three patterns are described which indicate the rate of decline of intraocular volume as a function of intraocular pressure. The first two patterns (monophasic and biphasic) probably indicate an effect due solely to aqueous outflow. The third pattern (polyphasic) most probably represents the summation of two factors: aqueous outflow and a changing blood volume.

Ophthalmology Branch (14).

Acknowledgment

I wish to thank Dr. L. von Sallmann for his helpful criticisms and Mr. J. G. Brown for his technical assistance.

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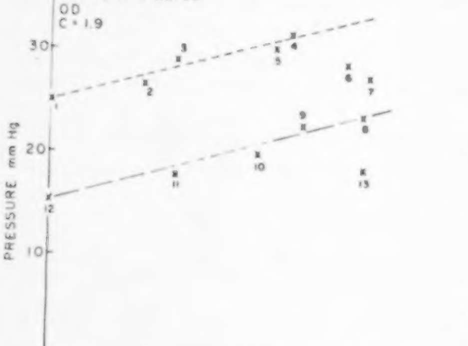
Discussion

Dr. Robert A. Moses (Saint Louis): Dr. Macri's interesting paper points up the fact that in the cat, as in other species investigated, we have much to learn about the hydrodynamics of the eye.

The work done in our laboratory by Dr. Constant and Dr. Becker was almost all at low pressure levels with a view to maintaining as close to physiologic conditions as possible, since these investigators found that if one raised the intraocular pressure too high—hardly above 30 mm. Hg in the cat—one got apparent inhibition of secretion (slide 1), and further determinations gave comparable C values but with a lower P_o intercept.[1] Similar observations were made by Bárány.[1] These experiments were perfusion studies at multiple constant pressure levels, and all tend to show that study of the aqueous outflow is a tricky business.

As a matter of fact, Constant and Purnell[2] showed that merely inserting a blocked needle into a cat's anterior chamber altered the outflow if left there for a matter of hours. Purnell[3] also found that a cat eye enucleated under anesthesia has a lower facility of outflow than if the animal is killed first and then the eye is enucleated.

Of the species they have investigated so far, Becker and Constant[4] found that only the guinea pig and man have stable enough secretion and outflow patterns so that one can obtain uniform, repeatable determinations on the same in situ eye by perfusion.



Slide 1

Dr. Macri points out that in the monophasic pattern the average facility of outflow in the cat is 0.28 mm.³/min./mm. Hg. The lower limb of the biphasic pattern averaged 1.8 mm.³/min./mm. Hg, which is much closer to Becker and Constant's perfusion values (in vivo 1.54, in vitro, 1.62).[4]

There is a question I would like to ask Dr. Macri. Does a given in vivo eye show the same outflow pattern on repeat determinations? Another question is whether Dr. Marci finds the same outflow pattern with ascending pressures as with descending pressures.

Dr. Frank J. Macri (closing): I would like to thank Dr. Moses for discussing this paper, and for the very fine points he has brought up.

We have, of course, considered whether elevating the intraocular pressure influences the inflow rate. This problem was dealt with most recently by Dr. Langham in his current review in the *Journal of Physiology;* preliminary work which we have been doing also indicates that inflow is a function of the intraocular pressure. It has to be presumed, however, that suppression of inflow by the elevated pressure would manifest itself by an increase of the outflow rates. At no time were the outflow rates at elevated pressures faster than those at the lower pressure.

We primarily determined the individual outflow patterns so that the effects of drugs on outflow could be better evaluated. The technique which we elaborated represents a simple method for the determination of facility of outflow and proves to be a valuable tool for such investigations.

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Probably there are errors inherent in the method, but the reproducibility of the results is quite good. Occasionally the monophasic outflow pattern shifts to the biphasic pattern.

Preliminary observations indicate that such a shift is brought about most frequently by the administration of Diamox and that this phenomenon can be reversed by the use of arterenol. Thus these two outflow patterns may be influenced by drug action.

I am afraid I do not know how to determine outflow on the basis of ascending pressure increments. The procedure used is not to be considered a perfusion experiment. It consists of the recording of spontaneous falls of the pressure as a function of time.

THE EFFECTS OF BETA RADIATION ON AQUEOUS SECRETIONS IN RABBITS

Frank S. Schiff, M.D.
Alhambra, California

It has been noted in the past by Becker, Cibis, and others that certain types of radiation to the eye caused temporary reductions in intraocular pressure; for example, 1,300 r of total ocular X-ray exposure would induce a hypotony for eight days after irradiation.[1] Such X-ray therapy has been used for temporary relief of pain and pressure, in cases of absolute glaucoma, although the scattering effects of the rays, as well as the damage inflicted upon retinal vessels and lens, discouraged this treatment for other types of glaucoma. A more localizing effect of irridiation on the ciliary body would, therefore, be more desirable.

Theoretically, an ideal type of radiation would be one which affected only the ciliary processes without ionizing those tissues in front of or beyond this target. Both the protons emitted by the cyclotron and the electrons coming from a linear accelerator have such pinpointing properties. According to the Rutherford-Bragg distribution curve, protons show peak ionization close to the end of their range.[2] However, this method would probably present great technical difficulties in accuracy of the aim as far as ocular use is concerned. Monoenergetic 2.5 mev electrons from a Van-de-Graaf machine delivers 100-percent radiation to a depth of two to four mm., but, unfortunately, also brings more than 50-percent to the outer surface and to the lens.[3]

The pure beta radiation from a Strontium^{90} applicator, on the other hand, is far from an ideal agent. In placing this instrument on the sclera over the ciliary body, 100 percent of the radiation affects the outer surface, while about 12 to 20 percent reaches the ciliary processes (1.5 to 2.5 mm. from the surface in rabbits), and five percent the lens equator.[2] Thus, it has been found that corneal ulcers, with or without perforation, may occur when beta dosages over 35,000 rep are directed to any given area. These complications usually occurred from 50 to 250 days after exposure. Corneal pannus, scleral telangiectasias, iritis, and cataract may also follow.[4]

Strontium^{90} beta radiation was nevertheless used for this initial experiment because it is technically easy to utilize.

Method

Normal adult albino rabbits were used. Intraperitoneal Pentobarbital anesthesia was given for every procedure, including tenometries. Initially, a pressure reading was done on each eye. One of the rabbit's eyes was used as a control and the other eye treated as follows: as Strontium^{90} applicator with an emitting surface of 13-mm. diameter was placed on the globe with two mm. of diameter extending over the cornea and the rest over the sclera. Ten mm. of diameter was in actual contact with the globe. The applicator, which was calibrated at 13.2 rep per second, was left on a given area until the standard 40,000 rep area dose had been achieved (20,000 rep was given to an area in only one instance). No more than 40,000 rep was given

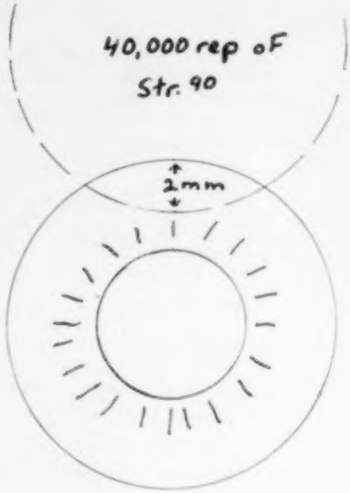

Fig. 1 (Schiff). Method of application.

to any area. In one series, a 0.5-mm lead shield with a 3.0 by 10-mm. opening was wrapped around the emitting surface of the applicator. No eye received more than one course of treatment.

Periodic tensions were taken of both the irradiated and control eyes. Outflow studies were performed with an electric tonometer.

RESULTS (See figs. 2 through 6)

When a total of 60,000 rep was given to an eye, all significant hypotony had disappeared in about 55 days, on the average. Aqueous outflow appeared to be normal after five months. A total of 80,000 rep has produced a lasting hypotony to date, although to a lesser extent in shielded eyes. Aqueous outflow was abnormally low in the unshielded eyes after four months. Eyes treated with

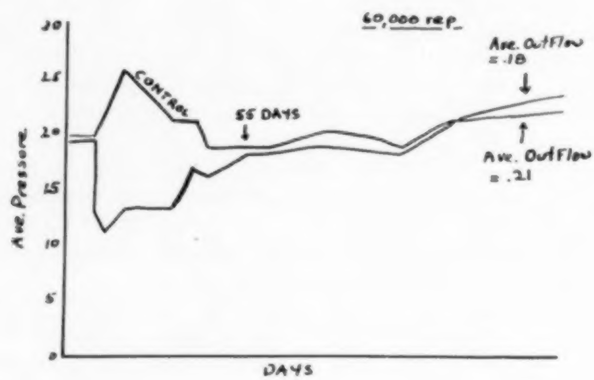

Fig. 2 (Schiff).

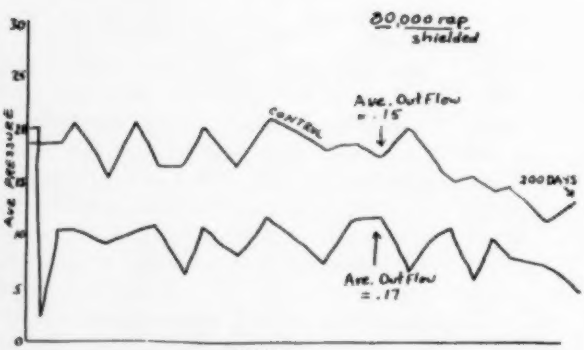

Fig. 3 (Schiff).

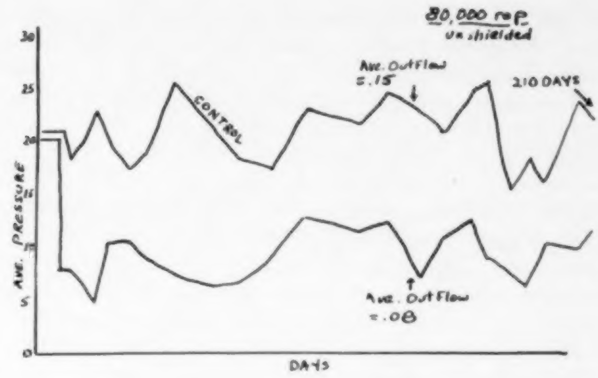

Fig. 4 (Schiff).

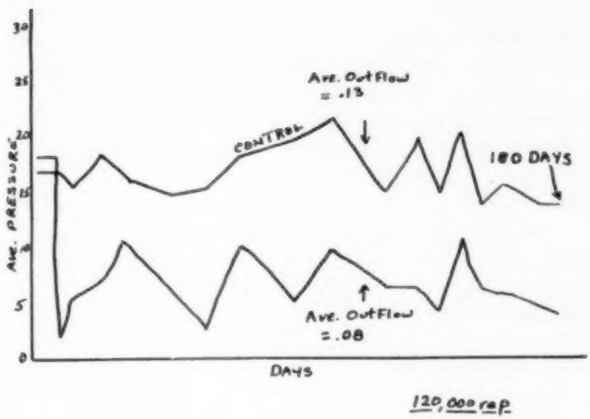

Fig. 5 (Schiff).

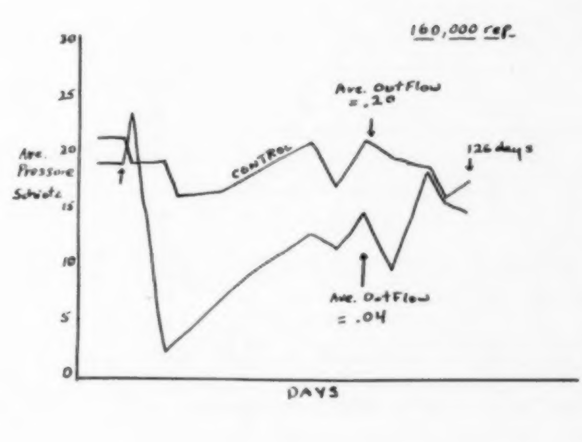

Fig. 6 (Schiff).

120,000 rep have maintained hypotony to date, 108 days after radiation. Average aqueous outflow was abnormal after three months.

The eyes having received 160,000 rep present a different picture. Instead of the abrupt hypotony which actually began at the end of irradiation in the previous eyes, one finds here an initial rise in tension for the first week. This was coincident with a severe iritis. Later, a profound and then a very moderate hypotension followed. Aqueous outflow was markedly impaired after three months. Histologic sections showed necrosis of the stroma and secretory epithelium in irradiated portions of the ciliary body.

Complications

Eyes having received a total of 80,000 rep or more showed permanent iris atrophy as well as permanent corneal and iris vascularization within a few weeks after irradiation. Conjunctivitis abated within four weeks in the eyes treated with 80,000 rep. All treated eyes showed mild permanent scleral telangiectasias.

Eyes treated with 120,000 rep had a moderate iritis lasting about two months. The iritis in eyes having received 160,000 rep was severe, with corneal edema, but this did not last more than two months. Virtually all of the treated eyes showed some peripheral choroiditis and occasionally a peripheral preretinal hemorrhage (even in one shielded eye). The eyes treated with 160,000 rep suffered severe retinal and vitreous hemorrhages within two months after irradiation.

Mild equatorial lens changes were noted in most treated eyes (80 to 160,000 rep) after two months.

Comments

Only a few conclusions can be drawn from this experiment to date. If Strontium^{90} were to be used clinically, it would have to be restricted to aphakic glaucoma with application preferably placed in areas which already display peripheral anterior synechiae. In order to minimize the chorioretinal reactions, it would also seem preferable to use an applicator with a far smaller emitting surface. One of the advantages of beta radiation over the classical cyclodiathermy lies in the former's greater accuracy of dosages, reducing the variability of the effects on the ciilary body.[5]

It appears to be difficult to induce phthisis by means of very high radiation doses. Excessive hypotony is counteracted by a decrease in aqueous outflow.

There are, of course, many unexplored aspects to this problem such as applications being given at different time intervals or the use of other radiation sources.

Summary

Beta rays from a Strontium^{90} applicator, when applied across the sclera to the ciliary body, will produce a hypotony if the total dosage to the eye is at least 80,000 rep.

This experiment is still in progress.

1237 *East Main Street.*

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Discussion

Dr. Peter C. Kronfeld (Chicago): These are preliminary experiments in search of a technique of ionizing radiation that will lower the intraocular pressure with a minimum of side effects.

The doses Dr. Schiff has used are large. If you will recall, the average therapeutic dose of beta radiation in man is about 16,000 rep. With the applicator which Dr. Schiff used and which was calibrated by Wilson and Wilson a few years ago, the minimum inflammatory dose is 35,000 rep. All of Dr. Schiff's doses were greater than that. Wilson and Wilson also found that doses of 100,000 rep in the rabbit almost invariably caused serious corneal complications. So, the doses that Dr. Schiff has used are large, indeed.

When I first read the title of his paper I was looking forward to a situation which might lend itself to tonographic analysis. I don't believe Dr. Schiff has enough data here to make such an analysis.

All that can be said at the moment is that the radiation effects on the fluid-producing system as well as on the fluid-eliminating system were very massive. There is also a strong possibility of a change in the rigidity of the sclera. The effects that he obtained with the smaller doses, 40,000 and 60,000 rep, are possibly effects purely on the ciliary body.

The question arises: Is this approach a promising one? I would think it is. Smaller applicators could be tried over areas farther back since damage to the anterior retina would be less serious than damage to the chamber angle, which is the lesson we have learned from cyclodiathermy. Smaller doses applied to the posterior portion of the ciliary body, with the effects more thoroughly watched than Dr. Schiff was able to do in this first series, might lead to a condition of practical or investigative value—investigative in the sense that we would have produced a less efficient aqueous-producing system on which certain other studies could be made. I feel that studies of this type should be continued.

Dr. Frank S. Schiff (closing): I want to express my appreciation to Dr. Kronfeld. I have very little to add to what he has said. I agree completely with what he has said. Especially do I agree about the point on the changes in the scleral rigidity.

We will endeavor to have the tracer laboratory make a smaller applicator and to use smaller dosages in future experiments.

TONOMETRY*

The pressure-volume relationship in the intact human eye at low pressures

Robert A. Moses, M.D., and Ahti Tarkkanen, M.D.
Saint Louis, Missouri

Introduction

Friedenwald formulated ocular distensibility as: $\log P_2/P_1 = E(V_2 - V_1)$, in which P_1 and P_2 are different intraocular pressures, V_1 and V_2 are the corresponding ocular volumes, and E is a constant characteristic of the individual eye ("coefficient of scleral rigidity").[1] This formulation has recently been questioned by Macri,[2] working with cat eyes, Perkins[3] using rabbit eyes, and McBain[4,5] and Macri[6] measuring human autopsy eyes. These workers injected small quantities of fluid and measured the consequent change of pressure. It seemed pertinent to utilize another method for obtaining comparable data in living, intact human eyes.

Theory

The applanation tonometer is an attractive instrument for displacing calculable volumes by measurable forces. When the cornea is flattened, the volume displaced (V) is given by $V = \pi h^2 (r - h/3)$ in which *r* is the curvature of the sphere (radius of corneal curvature) and *h* is the altitude of the seg-

* From the Department of Ophthalmology and the Oscar Johnson Institute, Washington University School of Medicine. This investigation was supported in part by a research grant, B-621, from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, Public Health Service. The research relating to this study was also financed in part under a grant to Washington University School of Medicine made by the Alfred P. Sloan Foundation, Inc. The grant was made upon recommendation of the Council for Research in Glaucoma and Allied Diseases. Neither the foundation nor the council assume any responsibility for the published findings of this study.

ment. The latter is given by the formula $h = r - \sqrt{r^2 - R^2}$, in which R is the radius of the base of the segment ($2R$ = diameter of the flattened area).[12] Adolph Weber[7] in 1868 and independently Priestley Smith[8] in 1879 and Imbert[9] in 1885 established the relationships of tonometry in general as: $P_t = F/A$, in which P_t is the pressure in the eye during tonometry (gm./cm.^{2}), F is the force of the tonometer (gm.), and A is the area supporting the tonometer (cm.^{2}).

In brief, if the force with which a plane is applied to an eye, the radius of curvature of the cornea, and the flattened area are known, one can compute the volume of ocular contents displaced from beneath the flattened dome of cornea (V) and the intraocular pressure during tonometry (P_t).

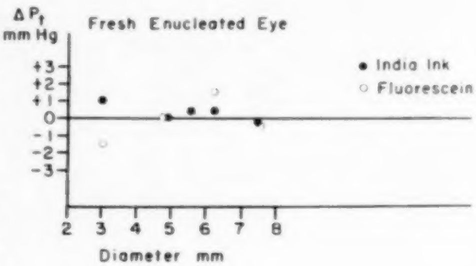
However, Goldmann and Schmidt[10] have pointed out that the structure of the cornea offers some resistance to deformation, while the surface tension of the tears attracts the plane to the cornea if the plane is wettable. The forces acting in opposite directions may not balance and this may necessitate the application of a correction.

Method

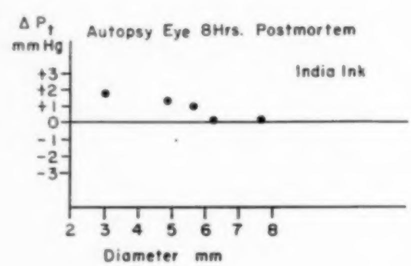
The prism supplied with the Goldmann applanation tonometer has an applanation diameter of 3.06 mm. In addition to this we constructed similar methacrylate (Plexiglas) prisms of 4.89, 5.67, 6.25, and 7.63 mm. applanation diameter. However, the 7.63 mm. diameter prism was not practical for clinical measurements. The prisms were mounted in the Goldmann tonometer and measurements were made in the usual fashion. The scale of the tonometer is in grams of force.

Corneal curvature was measured with a keratometer (Bausch & Lomb). Applanation measurements were made first with the Goldmann prism and then with our prisms. Each measurement was made as rapidly as possible and the series of measurements with the different prisms was accomplished at widely spaced time intervals (no less than 45 minutes apart) in order to avoid tonographic effect and the effect of flow of corneal substance.[10] Additional force was added to the tonometer arm as necessary by means of calibrated weights. (The calibration bar supplied by Haag-Streit is useful for this purpose.)

The prisms were calibrated against enucleated human eyes in an attempt to compare the calculated tonometric pressure with manometric pressure, and calculated volume displacement with measured volume displacement. For this purpose we employed a



1a



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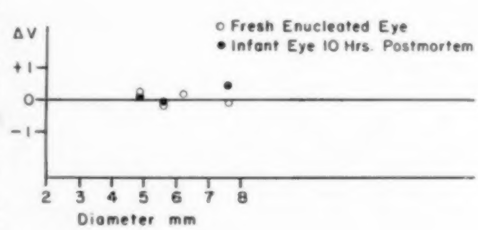

1c

Figs. 1a, 1b, 1c (Moses and Tarkkanen). Calibration of applanation prisms. $\Delta P = F/A - P_t$, in which F/A is computed and P_t manometric pressure. ΔV is the difference between computed and measured volume (mm.^{3}).

perfusion apparatus similar to that used by Becker and Constant,[11] allowance being made for the capillary force of the meniscus in the pipette. In enucleated eyes the corneas rapidly stained with fluorescein so that it became impossible to differentiate between cornea and meniscus. In order to recognize the meniscus, india ink was used in contrast to the green fluorescein-stained cornea. The surface tension effects of india ink and fluorescein are of the same order of magnitude[13] (fig. 1a).

Results

Representative calibration studies on fresh human eyes are shown in Figures 1a, 1b, and 1c. On the basis of measurement obtained from nine eyes we found no consistent deviation from calculated pressure and volume values. Therefore no correction factors were applied to clinical measurements.

In Figure 2 (a-c) are plotted the averages of the calculated tonometric pressures and corresponding calculated displaced volumes in normal eyes of volunteers. It is seen that these results fall on reasonably straight lines on semilog plots.

The results obtained in glaucomatous eyes are ploltted in Figure 2 (d-j) and also present reasonably linear plots.

Discussion

Friedenwald[12] pointed out that Kalfa's data with applanation tonometers may be

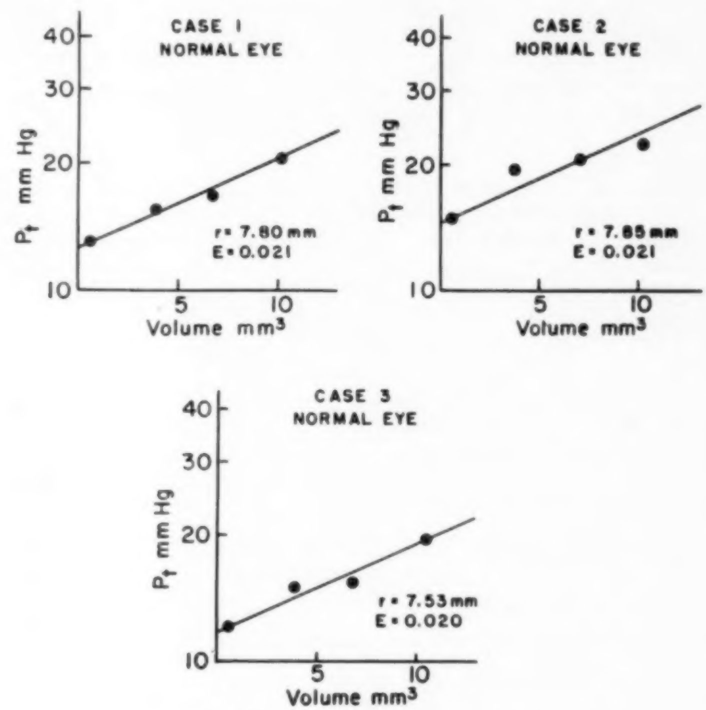

Fig. 2—a, b, c (Moses and Tarkkanen).

Fig. 2a-j (Moses and Tarkkanen). Clinical measurements in human eyes in situ. In all plots x-axis is a linear scale, y-axis a logarithmic scale. r = radius of corneal curvature; E = value of ocular rigidity.

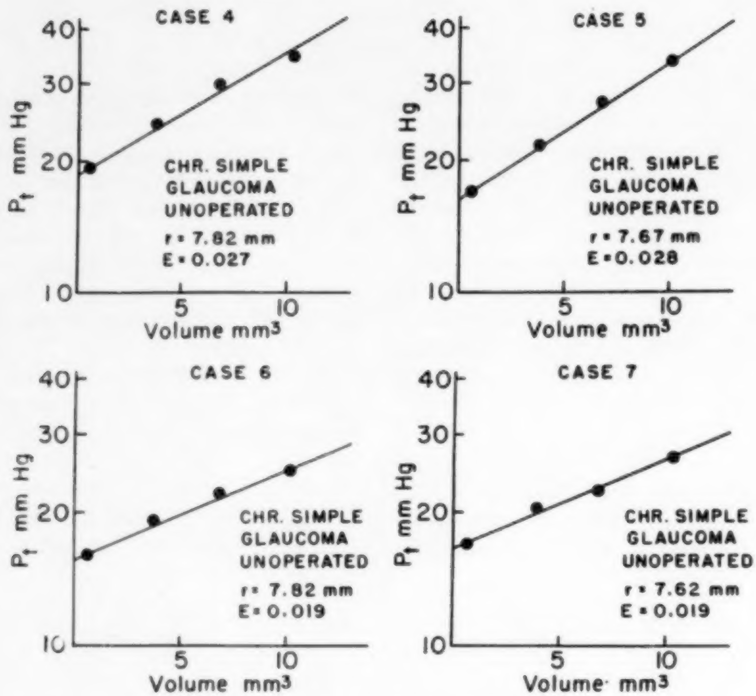

Fig. 2—d, e, f, g (Moses and Tarkkanen).

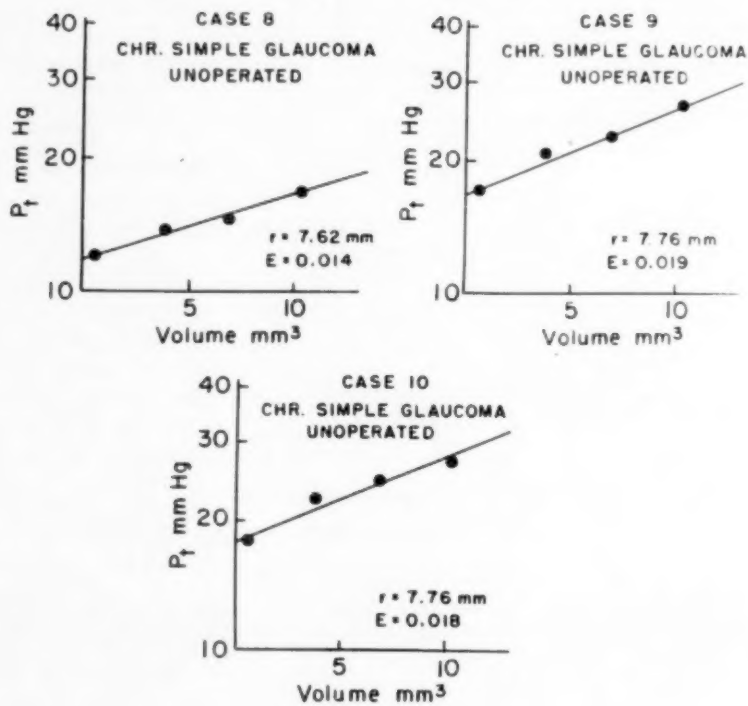

Fig. 2—h, i, j (Moses and Tarkkanen).

plotted on a log P_t versus V nomogram similar to that which he designed for the Schiøtz tonometer resulting in similar straight-line plots.

Goldmann and Schmidt (fig. 3) found that for applanation between three and five mm. diameter there was a sloping linear relationship between ΔP and diameter (ΔP = computed pressure minus manometric pressure). We have found no such consistent trend between 4.9 and 7.63 mm. diameter (figs. 1a and 1b).

Applanation at large diameters becomes difficult and uncertain due to the fact that the area of flattening increases as the square of the radius of the flattened area, and the volume displaced increases approximately as the cube of the radius of applanation. Thus, the measure of the flattened area becomes exceedingly critical.

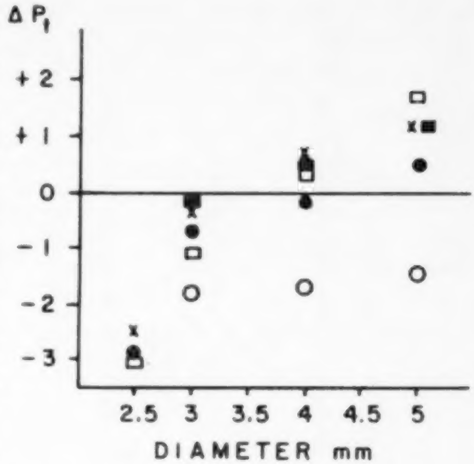

Fig. 3 (Moses and Tarkkanen). See text. (From: Goldmann and Schmidt: Ueber Applanationstonometrie. Ophthalmologica, 134:221, 1957.

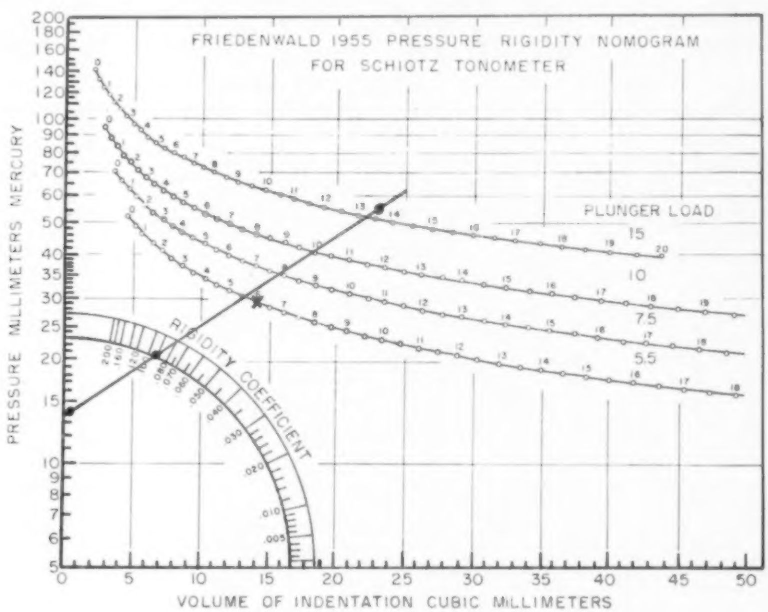

FIG. 4a (Moses and Tarkkanen). x = Schiøtz tonometer; o = Applanation. (See Addendum.)

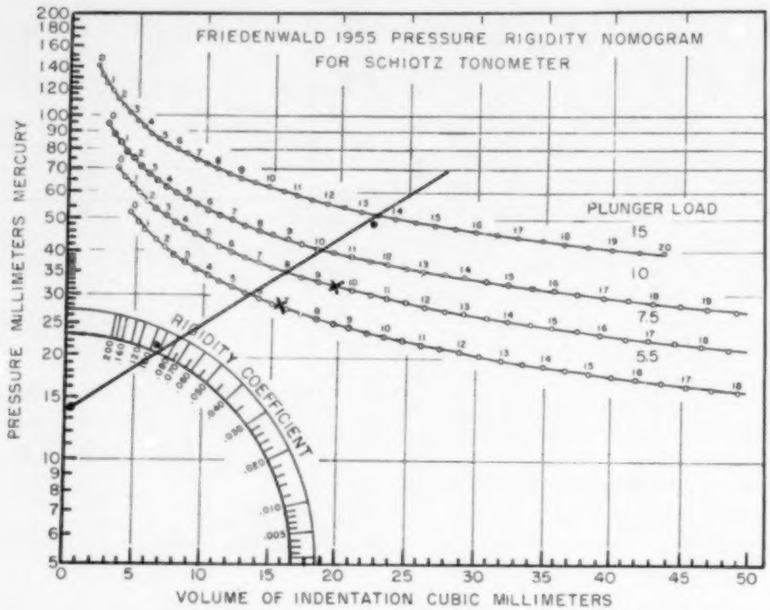

Fig. 4b (Moses and Tarkkanen). x = Schiøtz tonometer; o = Applanation. (See Addendum.)

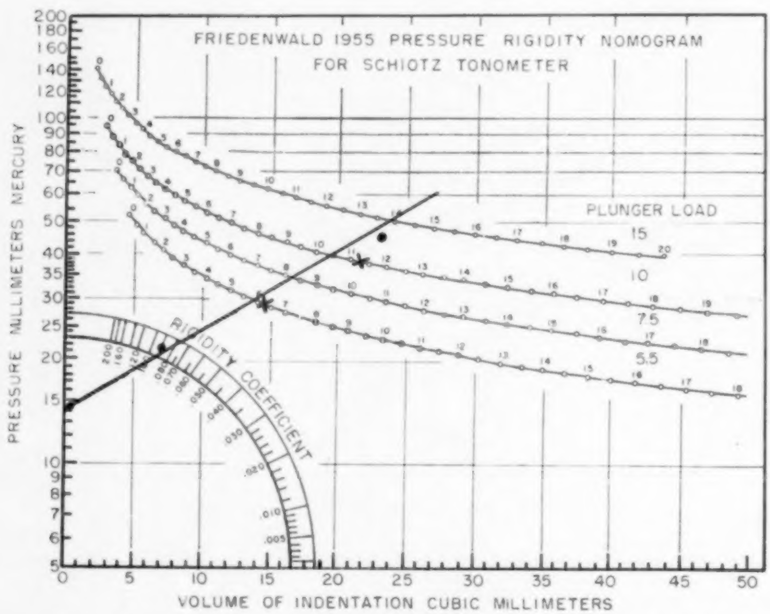

Fig. 4c (Moses and Tarkkanen). x = Schiøtz tonometer; o = Applanation. (See Addendum.)

Summary and Conclusions

Applanation tonometry with different sized applanation areas appears to confirm the Friedenwald formulation of the linear relationship between corneal indentation volume and the logarithm of the tonometric pressure in the living human eye.

640 South Kingshighway (10).

Addendum

Since this paper was presented, we have been able to obtain several measurements with the 7.63 mm. prism. These are presented in Figures 4a, 4b, and 4c in conjunction with the 3.06 and 5.67 mm. measurements, and with Schiøtz tonometer readings for comparison.

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Discussion

Earle H. McBain (San Rafael, California): Most measurements of pressure-volume relationship, or ocular rigidity, in the eye have been made on dead eyes. It has always been questionable whether these results could legitimately be applied to living human eyes. The authors' work with the applanation tonometer represents an attempt to make rigidity measurements on living eyes, and this is certainly to be commended.

It should be pointed out, however, that this method is subject to some of the same errors inherent in the use of indentation tonometers such as that of Schiøtz for measuring ocular rigidity, in that both pressure and volume are determined indirectly by calculation after making certain assumptions. It is true that the deformation of the cornea produced by the applanation tonometer has a more regular shape, but in attempting to correlate calculated with measured pressure the authors found a rather wide scatter. The three-mm. flattening has been rather carefully worked out by Goldmann, but readings of pressure with the larger areas of applanation are probably subject to more errors.

In calculating volume of intraocular fluid displaced by the tonometer it is assumed that there is no indentation of the posterior hemisphere of the globe and also that the curvature of the cornea adjacent to the flattened area is not altered. Although these factors may not be important with the three-mm. prism, one would think that they might be sources of inaccuracy with the larger prisms.

The only way to accurately measure rigidity is to determine the actual change in pressure produced by the injection of a measured volume of fluid into the eye. So far this has not been done in the living human eye for obvious reasons. This procedure has often been done on animal and enucleated human eyes with variable results. In our laboratory, using a sensitive strain gauge, recording potentiometer and microburette on enucleated human eyes, with the cannula passing through the posterior chamber into the pupil, we have repeatedly found an inverse relationship between the semilogarithmic coefficient of Friedenwald and the intraocular pressure. This means that, on the semilogarithmic plot, as shown by the authors here today, a curve, convex upward, is seen. Formulas fitted to the experimental data have shown a deviation of less than one percent of the calculated values from the measured values. In many of these eyes the rigidity (as expressed by the semilog formula) has been as much as 100 percent greater at a pressure of 10 mm. Hg than the rigidity at

50 mm. Hg. Therefore, if this can be shown to be true in living eyes, some revision of the tonometer tables will be necessary.

In five of the cases just presented (2, 4, 5, 6, 10) a curve, convex upward, is seen, which agrees with our strain gauge findings. In the other five a zig-zag arrangement of the four points occurs with the second point lying above the line and the third below. I wonder if the authors are justified in concluding that these findings confirm the semilogarithmic relationship between pressure and volume. Perhaps it would be better to say that they are not incompatible with such a relationship.

In any case I believe that the problem is not settled and perhaps will not be until actual pressure-volume measurements with a cannula are carried out in living human eyes. It is hoped that the authors will continue their work in that direction.

DR. ROBERT A. MOSES (closing): I thank Dr. McBain for his kind discussion. I agree with his criticisms in full. This complex problem of scleral rigidity will not be solved until we do work with living human eyes.

A CONTRIBUTION TO THE ELECTRORETINOGRAM OF RETINITIS PIGMENTOSA*

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The electroretinogram of retinitis pigmentosa has been discussed repeatedly in ophthalmic literature (Karpe,[1] Riggs,[2] Jacobson,[3] Franceschetti,[4] François,[5] Burian[6]). It has been described as extinct in true cases of the disease. This would indicate that the retina is incapable of developing a potential in response to light stimuli, independent of the progress of the disease. Moreover, this implies that the electric reactions are extinct, even in the presence of peripheral vision. Such a phenomenon is difficult to explain on the basis of present day knowledge of the electroretinogram.

One must then define the meaning of the term, retinitis pigmentosa:

1. *The characteristic clinical picture.* The disease is described as a bilateral, symmetric, progressively blinding disease, beginning with loss of night and peripheral vision and progressing to mere light perception or less. The disease often times appears in childhood and very often is familial. (The hereditary picture is difficult to evaluate, however, for there are six accepted ways for the disease to be transmitted.)

2. *The ultimate fundus picture* is notable by the presence of pigment clumps or spicules, attenuation of arterioles, and waxy pallor of the optic discs.

3. *The visual fields* usually demonstrate losses in the midperiphery—or ring scotoma—progressing peripherally and centrally until only a small central area of vision exists.

This series consists of over 38 cases, complaining of visual difficulties, in association with the typical bone spicules of pigment in the retina.

ELECTRORETINOGRAPHIC METHOD

A routine protocol was followed in each case:

1. The pupils are dilated with 10-percent neosynephrine.

2. The method of examination is explained to the patient as he sits in the examining chair (relaxing type, contour) for 15 to 20 minutes in reduced illumination.

3. After local anesthesia, lacrilens type contact lenses (in three sizes) with silver wire electrodes are placed over both corneas.

* From the Kresge Eye Institute. Presented before the East Central Section of the Association for Research in Ophthalmology, January 6, 1958.

The electrodes are connected to a headband which contains reference and ground electrodes on the forehead.

4. The patient is positioned comfortably, facing the reflector of the Grass Photic Stimulator at a distance of one meter and fixating a red light within the reflector.

5. Records are taken from a double beam cathode ray oscilloscope by Polaroid Land Camera. The stimulus triggers the cathode ray beam adjusted to a sweep speed of 20 msec./cm. 200 microvolts equal 1.5 inch on the screen surface. The time constant of the system is routinely 300 msec. and in special tests approximately one second. The high frequency response is 50 percent at about 200 cycles.

6. The stimulus is obtained from a Grass Photic Stimulator. First, the patient receives a short series of stimuli, several seconds apart in dim-room illumination, intensity setting No. 1 of Grass stimulator; no filter. After total darkness for five minutes, stimuli at settings 1, 4, and 16 are tested and recorded singly and in series. No filter is used. With intensity 16, the interval between single flashes is one minute. Maximum intensity (16) of the Grass flash is 1,500,000 candle power at 10 inches according to specification; duration is 10 microseconds. A Grass red filter (of 10-percent transmission at 600 millimicrons, 50 percent at 620 millimicrons) is then interposed, and, at intensity 16, several stimuli are tested and recorded. Flicker of 20/second, followed by 50/second, at intensity 4, no filter, is thereafter tested and recorded.

7. With each part of the experiment, superimposed photos are made with the Polaroid Land Camera.

8. The same technique is followed in each case utilizing similar oscilloscope, amplifier, and stimulator settings. With each case calibration signals indicating amplification and sweep speed are recorded.

Case reports*

Case 31

S. R. L., a 41-year-old white woman, first noted loss of vision one year ago. She has one sister with clinically diagnosed retinitis pigmentosa and her father was blind at his death from clinically diagnosed retinitis pigmentosa (A. D. R., Sr.) Vision with correction: R.E., 20/20—4; L.E., 20/30 —1.

Ophthmaloscopy showed the typical bone spicule formaion except not much was noted and the discs and vessels appeared normal. Visual fields and the electroretinogram of this patient are illustrated in Figure 1.

Case 32

M. L., a 50-year-old white woman, noted loss of vision for some years and further loss of vision in the past several years. This patient is the affected sister of Case 31.

Ophthalmoscopy showed the retinitis pigmentosa most marked in the inferior nasal quadrants (fig. 2). Visual fields had definitely decreased in the past one and one-half years.

An unaffected 53 year old sister of these patients was recently examined. Vision was reduced in one eye but this condition had existed all the patient's life. The vision in the other eye was correctible to normal limits. Ophthalmoscopy was normal, O.U. Visual fields were full. The electroretinogram was normal in every respect.

Case 33

L. G., a 46-year-old white man, had suffered loss of night and side vision since 1945, although spots had been noted in the right fundus since the age of nine years. No family history was obtainable. Corrected vision, R.E., was 20/40.

Ophthalmoscopy showed typical retinitis pigmentosa except more clumping of pigment than bone-spicule formation. The vessels looked fairly good and the discs were only slightly waxy. (See fig. 3.)

Case 34

A. B., a 47-year-old white man, stopped night driving in 1940. No difficulty was noted prior to that time. There was no familial history. Vision was: R.E., 20/30; L.E., +20/200.

Ophthalmoscopy showed classical retinitis pigmentosa (fig. 4). Biophotometer dark adaptation had a typical retinitis pigmentosa curve.

Case 35

P. F., a 47-year-old white man, had had visual loss for at least six years. Past medical history was negative except for long-standing hay fever. There was no family history. Vision, R.E., was 20/200.

Ophthalmoscopy showed arterial attenuation with perivascular pigmentation especially at the equators. Associated with this was severe macular change with some central pigmentation and edema. (See fig. 5.)

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* Cases 1 to 30 are listed in Table 1.

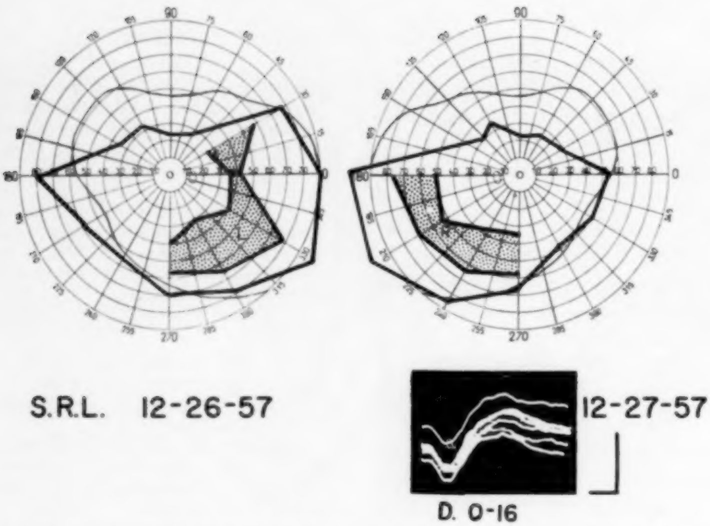

Fig. 1 (Ruedemann and Noell). Case 31 (S. R. L.). Visual fields from December 26, 1957, 3/330 white. The illustrated electroretinogram record (routine test [see method]) shows superimposed responses of both eyes. As in the following figures (2 to 8) the stimulus intensity is 16, no filter (0); the patient has been dark adapted (D) for five minutes previous to the test. Responses to three flashes are recorded; interval between flashes is one minute. The electroretinogram of both eyes has the same form (see also fig. 9). In this and the following figures the vertical line besides the tracing indicates 200 microvolts, the horizontal line denotes 20 milliseconds. The cathode ray sweep (running from left to right) is triggered by the stimulus (the same is the case in all other figures).

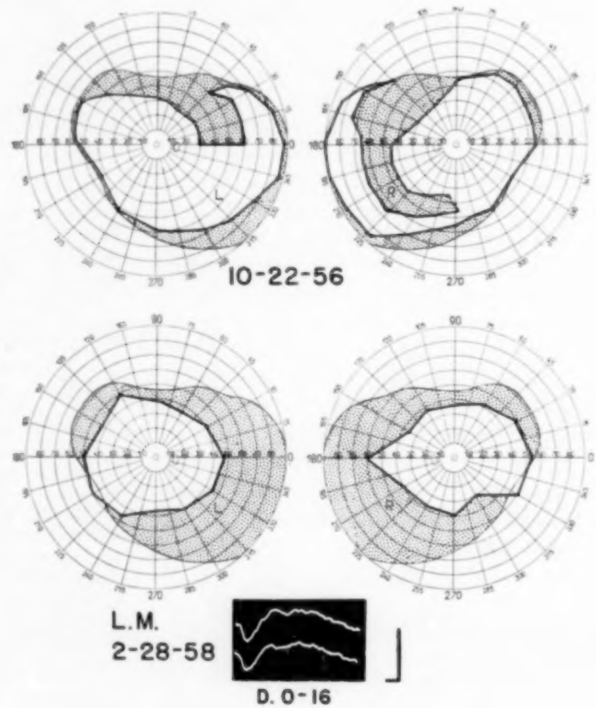

Fig. 2 (Ruedemann and Noell). Case 32 (L. M.). Visual fields, 3/300 white. Note the progress over one and one-half years. Electroretinogram is from a routine test performed at the same day as the visual field of the lower half of the figure. The upper electroretinogram tracing is from the right eye, the lower from the left, recorded simultaneously.

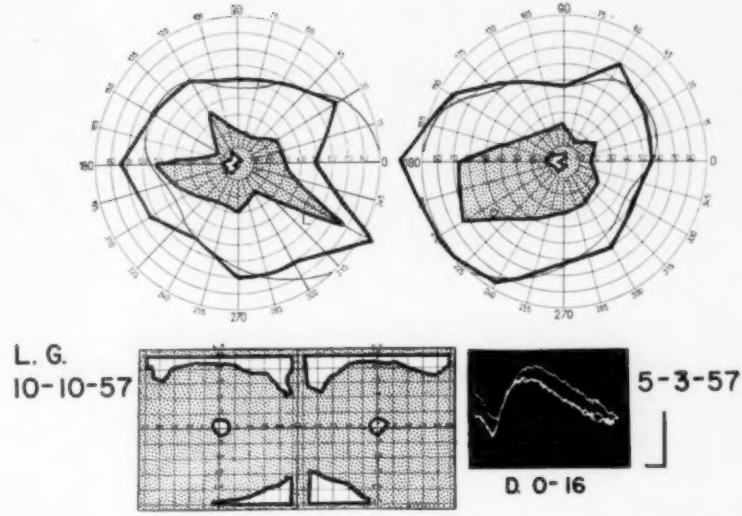

Fig. 3 (Ruedemann and Noell). Case 33 (L. G.). Visual fields: 3/330 white; 1/1,000 white. The illustrated electroretinograms are from one eye; three responses are superimposed (see also electroretinogram of fig. 9).

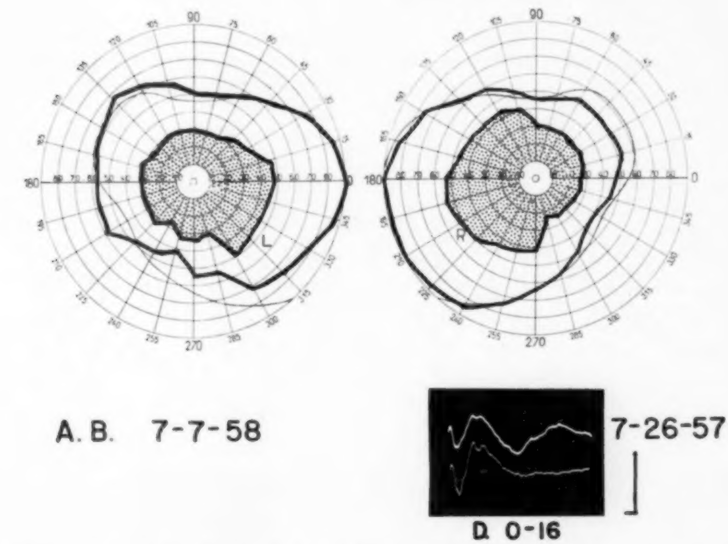

Fig. 4 (Ruedemann and Noell). Case 34 (A. B.). Visual fields: 3/330 white. Upper electroretinogram record (identified by greater cathode ray beam intensity) is from right eye, lower from left. (See also fig. 9.)

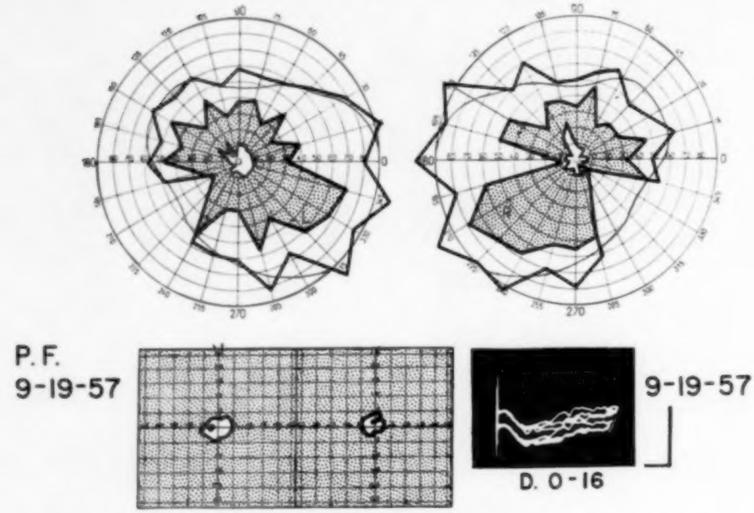

Fig. 5 (Ruedemann and Noell). Case 35 (P. F.). Visual fields: 3/300 white; 1/1,000 white. Electroretinograms are from both eyes (upper—right; lower—left). (See also fig. 9.)

CASE 36

W. M., a 38-year-old white man, first noted blurred vision in the morning one year ago coincidental with his first attack of severe hay fever. No family history was obtained. Vision with correction was: R.E., 20/40+2; L.E., 20/50.

Ophthalmoscopy showed typical retinitis pigmentosa. (See fig. 6.)

CASE 37

J. H., a 64-year-old white man, had had night blindness for many years but severe visual loss for only one year. There was no family history. Past medical history was noncontributory. Vision was: R.E., 20/200; L.E., 20/200.

Ophthalmoscopy showed peripheral pigmentary degeneration with changes in the macular areas,

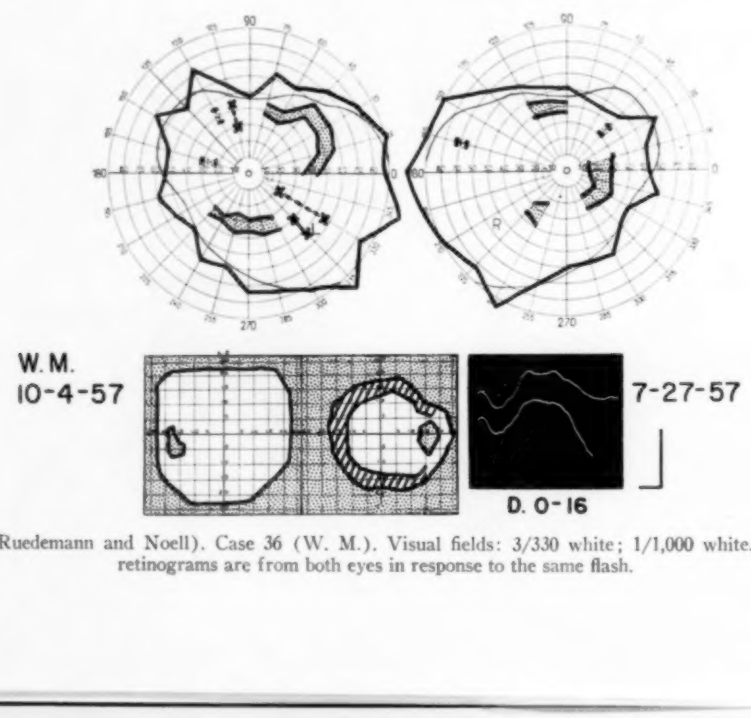

Fig. 6 (Ruedemann and Noell). Case 36 (W. M.). Visual fields: 3/330 white; 1/1,000 white. Electroretinograms are from both eyes in response to the same flash.

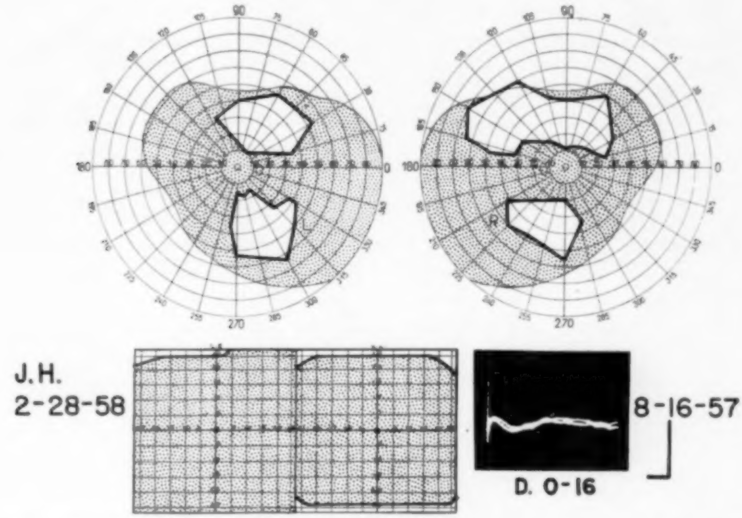

Fig. 7 (Ruedemann and Noell). Case 37 (J. H.). Visual fields: 3/300 white; 1/1,000 white. The electroretinogram is from right eye; left eye had a similar small response. Repeat test (four months later) showed the same small electroretinogram. Responses were only evoked with the two highest stimulus intensities tested (see method).

O.U. The macular changes were not pigmented, however. (See fig. 7.)

Case 38

D. S., a 30-year-old white man, noticed loss of peripheral vision three or four years ago and was required to stop night driving one year ago. His maternal grandmother was night-blind and had cataracts. His mother and her sisters were all right, as were his siblings (four girls, one boy). Vision was: R.E., 18/30; L.E., 18/30—1.

Ophthalmoscopy showed some bone spicule for-

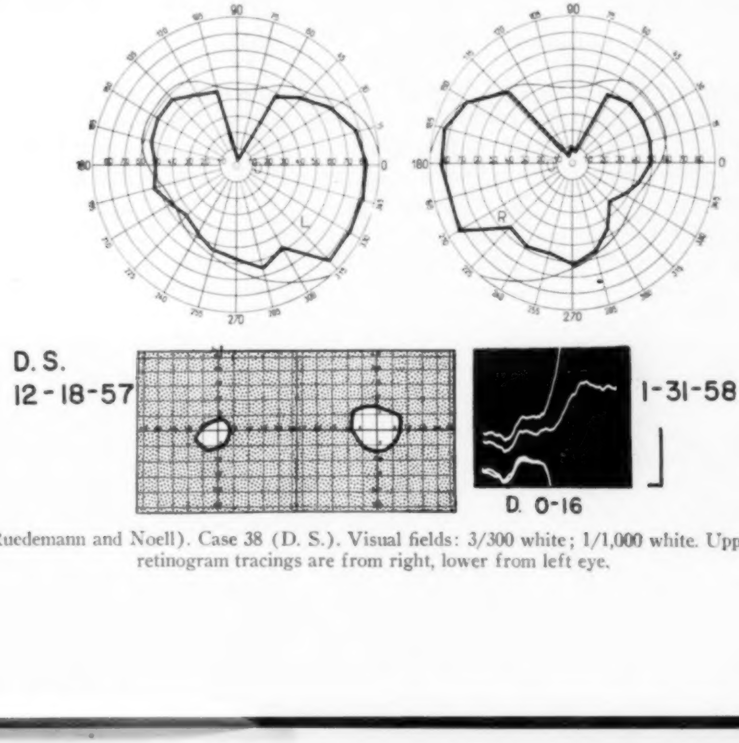

Fig. 8 (Ruedemann and Noell). Case 38 (D. S.). Visual fields: 3/300 white; 1/1,000 white. Upper electroretinogram tracings are from right, lower from left eye.

TABLE 1

DATA ON 30 CASES OF RETINITIS PIGMENTOSA WITH EXTINCT ELECTRORETINOGRAMS

No.	Name	Sex	Age/Race	Onset	Vision R	Vision L	Visual Field	ERG	Family History	Remarks
1	F. P.	F	34/W	Childhood	18/30	18/50	OU <5°	Extinct	Neg.	Def. peripheral field in '56
2	L. DeW.	M	49/W	30	20/40	20/40	<5°	Extinct	Neg.	Def. peripheral field in '45
3	A. B.	F	53/W	Unknown	20/200	20/200	<5°	Extinct	Neg.	
4	M. G.	F	57/W	Unknown	20/30 −2	20/40 +3	<5°	Extinct	Sister with Disease	
5	M. L.	F	55/W	20's	20/200	20/200	<5°	Extinct	Neg.	Post sub. caps.
6	A. L.	F	34/W	Childhood	LP	LP	Not enough vision	Extinct	See next	Parents 1st cousins
7	P. M.	M	36/W	20's	20/100	20/100	<5°	Extinct	Brother of above	Parents 1st cousins
8	C. M.	M	39/W	20's	20/50	20/100	<5°	Extinct	Brother of above	Parents 1st cousins
9	H. R.	F	35/W	20's	LP	20/200	Not enough vision	Extinct	Sister of above	Parents 1st cousins
10	A. O.	F	37/W	Unknown	20/50	20/50 −2	Not enough vision	Extinct	Sister of above	Parents 1st cousins. Maternal grandfather blind
11	M. N.	M	52/W	46	20/100	20/50 −2	N.T.	Extinct	Neg.	Thyroidectomy 3 years ago, cataracts
12	R. G.	M	25/W	10–15	20/40	20/30	5–10°	Extinct	Neg.	Post. central cataracts
13	S. E.	F	44/W	39	20/200	20/200	<5°	Extinct	Sister with disease	1 sister with disease
14	M. L.	F	49/W	Unknown	20/30+	20/30+	<10°	Extinct	Neg.	
15	L. S.	F	52/W	some years	20/50 −2	20/30	LE 15° RE markedly constricted	Extinct	Neg.	
16	R. C.	F	53/W	40's	20/100	20/50	<20°	Extinct	Neg.	
17	J. B.	M	80/W	Unknown	20/200	20/200	Not enough vision	Extinct	Neg.	
18	P. M.	M	43/W	14–16	LP	20/200	Not enough vision	Extinct	Neg.	
19	J. C.	M	28/W	Childhood	20/50	20/25	<5° OU	Extinct	Mother, 2 uncles, 2 sisters	
20	J. W.	M	37/W	Unknown	20/40	20/40	R 5–7° L <10°	Extinct	Neg.	
21	T. P.	M	18/C	Unknown	LP	20/200	<5°	Extinct	Neg.	
22	W. S.	M	41/W	14	LP	LP	Not enough vision	Extinct	1 Brother	
23	L. S.	F	25/W	4–5 yrs.	20/70 +3	20/40 +1	<5°	Extinct	1 sister	Deaf mute
24	R. B.	F	28/W	Childhood	LP	LP	Not enough vision	Extinct	Neg.	
25	L. R.	F	26/W	Childhood	20/70 —	20/200	<7°	Extinct	Neg.	
26	G. A.	M	22/W	Childhood	20/50 cc	20/50	5°–10°	Extinct	Brother with poor vision	
27	R. E.	M	43/W	Childhood	LP	LP	Not enough vision	Extinct	Neg.	
28	D. C.	M	30/W	Age 3	20/200	20/200	3–5°	Extinct	Neg.	
29	J. F.	M	36/W	Childhood	LP	LP	20–25° central scotoma OU	Extinct	Neg.	Macular degeneration
30	R. M.	F	42/C	Childhood	20/25	20/25	5°	Extinct	Neg.	

mation; vessels and discs were not markedly affected. (See fig. 8.) Biophotometer dark adaptation was markedly slowed.

RESULTS

The 30 cases listed in Table 1 had the frequently described *extinct* electroretinogram. The majority of these had a history of many years' duration, that is, the disease began in childhood. Every patient had reduced vision and a small visual field. The ophthalmoscopic picture was invariably typical. Several Negroes are represented in this group which also includes one family with five involved members out of 11 siblings. The parents were first cousins. All five involved members (cases 6, 7, 8, 9, and 10) and several siblings of the involved members were studied. The siblings appeared to have a normal electroretinogram.

The eight cases (31 to 38), listed separately, showed retention of the electroretinogram. Clinically these cases are notable in several respects:

1. Each case had a definite peripheral field. Mostly there was a midperipheral or ring scotoma.

2. Visual complaints were not noted in any case prior to the age of 25 years.

3. Although bone spicules of pigment were present in every case there was sometimes a question of amount as in Cases 31 and 38. The vessels were not always markedly attenuated nor were the discs particularly waxy.

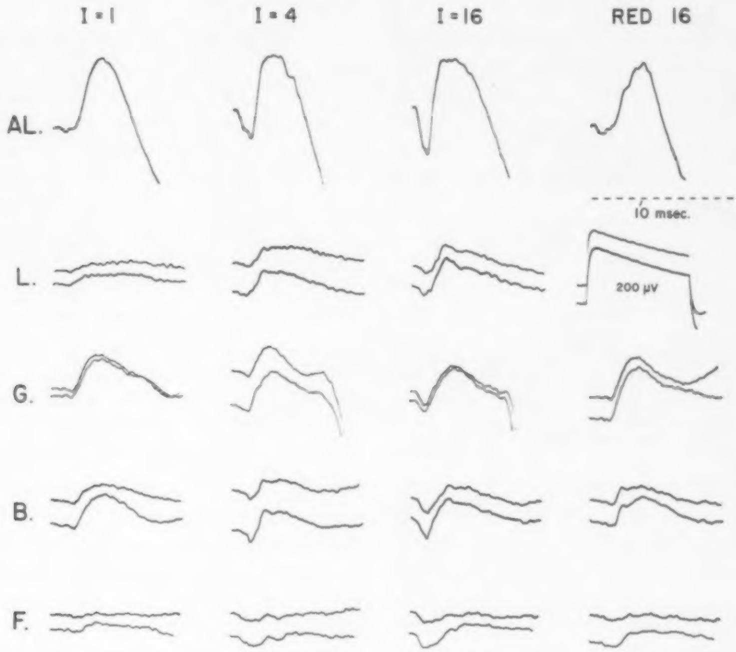

Fig. 9 (Ruedemann and Noell). Electroretinograms of Cases 31 (L.), 33 (G.), 34 (B.), and 35 (F.) recorded on the same day as a normal AL.). Each column shows the responses to the same stimulus. Intensities (I = 1) refer to the settings of the Grass Photic Stimulator (see methods). Filters are not interposed except for column marked Red 16 where the red filter (see method) is used at stimulus intensity 16. Amplification and sweep speed are the same for all records. The calibration record (200 microvolts) indicates the time constant and frequency response used in these tests. In all cases (but the normal) the electroretinogram of both eyes is recorded simultaneously; the upper tracing in each case is from the right eye. Each dash of the time line denotes 10 milliseconds.

4. Three of the eight patients had a family history of retinitis pigmentosa: Cases 31 and 32 are sisters and their father had retinitis pigmentosa. Case 38 had a night-blind grandmother.

Obvious electroretinogram retention is noted in these cases (figs. 1 to 8). In three cases (31, 33, 34) the total electroretinogram amplitude in response to maximal stimulus (after five minutes in the dark) is almost 200 microvolts; in two (32, 36) it is slightly more than 100 microvolts; and in three (35, 37, 38) it is between 30 and 80 microvolts. The electroretinograms of both eyes, simultaneously recorded, are very similar, practically identical. The changes, therefore, appear to be symmetric. With retesting over a period of several months, each case showed the same electroretinogram as recorded previously.

Although the electroretinogram was retained in these cases, it was invariably of abnormal form. Figure 9 shows a comparison of a normal (AL.) with cases 31 (L.), 33 (G.), 34 (B.), and 35 (F.). These cases were retested with the normal on the same day under the same conditions. It is evident that both a-waves and b-waves are reduced. They are not reduced equally, however. In L. and G. the b-wave appears better preserved than the a-wave; in F. the a-wave is more prominent.

Another abnormality is evident at high recording speed as illustrated in Figure 10.

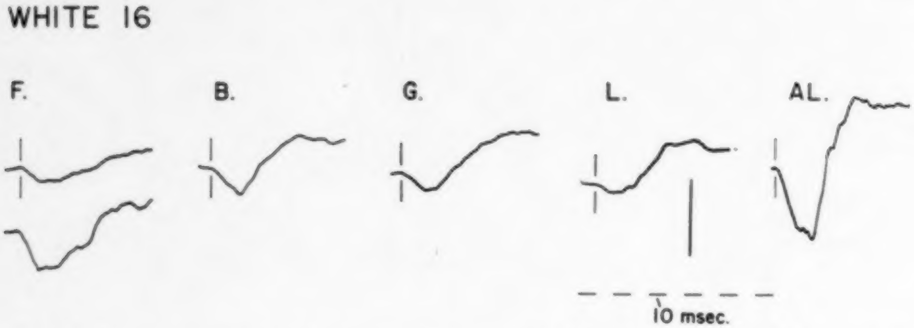

Fig. 10 (Ruedemann and Noell). Continuation of the tests of Figure 9 with sweep speed doubled. The distance between the start of each tracing and the vertical mark indicates the latency of the a-wave. The stimulus is maximal (16) in each case, no filters. The calibration line denotes 200 microvolts. Calibration is the same in each case except for the lower tracing of F. for which amplification was two and one-half times higher than in the other records.

Responses to maximum stimulus are recorded at twice the routine cathode ray sweep speed of the cathode ray. The vertical lines mark the onset of the a-wave. Since the stimulus triggers the sweep, the distance from the onset of the recording to the vertical line represents the latency of the a-wave. In comparison to the normal (AL.) this latency is significantly increased. In contrast, the time elapsing between a-wave start and b-wave appearance is not measurably greater than in the normal. These latencies and other phenomena of the retained electroretinogram in retinitis pigmentosa warrant further investigation.

Discussion

Any consideration of such a disease as retinitis pigmentosa is bound to raise argument.

First, the problem of diagnosis. In this series, besides the clinical data listed, nearly every patient received a thorough medical workup, including hospitalization in all but Case 38 of the eight with retained electroretinogram. No intercurrent disease of the liver, blood, and so forth, could be found. There was no syphilis or history of syphilis in any case. No virus infection was noted by history. Two cases with electroretinograms (35 and 37) had macular disease. They also had the lowest b-waves of the series. The consensus of ophthalmologic opinion (more than three) in each case was unanimous as to diagnosis. Dark-adaptation studies were performed on a few cases with retained electroretinogram and corroborated the clinical diagnosis. Three cases having a definite family history showed electroretinogram retention. Each patient claimed progression of the visual symptoms.

Second, the characteristic fundus picture of retinitis pigmentosa does not necessarily begin with a great amount of pigment, attenuation of vessels, or waxy pallor of the disc. Pigment proliferation and migration is a result of the progress of the disease; vascular attenuation follows upon reduced vascular demand. Therefore, the most typical case denotes a late stage of the disease.

Third, there is no question but that toxins and poisons may affect the rod cells to such an extent that the electroretinogram will diminish and the pathology picture of retinitis pigmentosa will develop. This has been described, for instance, in liver disease in humans and animals,[7] and in the rabbit and other species with various poisons.[8, 9] Characteristically, retinitis pigmentosa is a progressive disease. Those patients having a late onset might be expected to have a slower and milder course. It is notable that every pa-

tient with a retained electroretinogram had a late onset and has to date followed a mild course.

The following reasons may account for the reports in the literature that the electroretinogram is typically extinct in retinitis pigmentosa:

1. The reported cases may have had long standing disease beginning in childhood.

2. They may have had no peripheral field; therefore extensive loss of visual cell function may have existed.

3. Recording equipment may have been inadequate, for example, ink writer versus cathode ray oscilloscope.

4. Stimulus qualities were generally different from those of this study (see also Franceschetti[4]).

Skeehan, Passmore, and Armington[10] described a case with definite, though abnormal, electroretinogram. Karpe[1] also reported a single case with subnormal electroretinogram in a 65-year-old woman with 30-degree fields and a temporal rest. These cases would fit our observations well.

The demonstration of a definite electroretinogram in certain cases of retinitis pigmentosa also conforms with observations in hereditary visual cell degeneration in mice where the electroretinogram is present though abnormal while the disease develops and progresses (Noell[11]). The electroretinogram is extinct when all visual cells of the mouse have degenerated.

Summary

1. Thirty-eight cases of retinitis pigmentosa were studied.

2. The electroretinogram was extinct when no peripheral vision had remained. It was present when peripheral vision was evident.

3. When present, the electroretinogram was abnormal in form. The a- and b-waves were reduced in amplitude but not equally. In addition, the latency of the a-wave was increased.

4. In the series of cases studied to date the electroretinographic results would indicate a symmetric bilateral disease affecting primarily peripheral retinal elements.

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